

# PATHOLOGY

Also the Official Organ of the American Society for Experimental Pathology

**Intestinal Infarcts Produced by Forssman Antibodies**

*Seymour Levine and Bert Warren*

**Proteinuria Associated with Experimentally Produced Abscesses and Fever in Dogs**

*Robert D. Coyle, Roger Nichoff, Martin Rammner, and James Tanner*

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**Rheumatic-like Nodules Occurring in Nonrheumatic Children**

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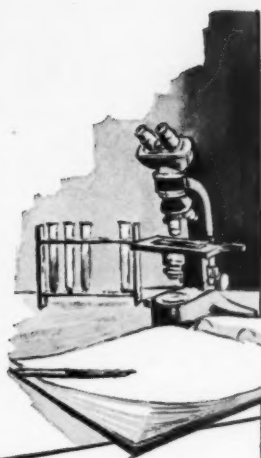
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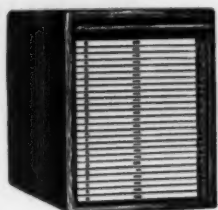
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# A.M.A. ARCHIVES OF PATHOLOGY

## Intestinal Infarcts Produced by Forssman Antibodies

SEYMOUR LEVINE, M.D., and BERT WARREN, M.S., Jersey City, N. J.

The possibility of an immunologic mechanism for intestinal disease has been subjected to experimental exploration in a number of ways, including active and passive sensitization, and Arthus, Auer, and Freund adjuvant techniques (reviewed by Kirsner and Elchlepp<sup>1</sup>). We chose to take advantage of the presence of Forssman antigen in guinea-pig tissues, and sought to induce intestinal injury by intra-arterial administration of Forssman antiserum. This procedure was analogous to that used by Forssman<sup>2</sup> and others to produce brain injury in the guinea pig (so-called "carotid syndrome"). The lesions resulting from injections into mesenteric arteries had all the characteristics of hemorrhagic infarcts.

### Methods and Materials

Female guinea pigs, weighing 250 gm., were anesthetized with ether or pentobarbital (Nembutal). Laparotomy was performed through a midline incision with aseptic precautions. The small intestine was exteriorized and kept moist with saline. Injections into the mesenteric arteries were made with a 30-gauge needle on a Luer-Lok syringe. The last branches before the terminal arterial arcades were chosen for injection. In the early experiments two sutures were passed through the mesentery around the artery (and its accompanying vein); the distal suture was tied; the injection was made into the artery retrograde, and, finally, the proximal suture was tied while the needle was still in place. This procedure, the same as that used in "carotid-syndrome" experiments,

minimized hemorrhage. The infarcts were in the intestinal territory supplied by the arterial branch which had its origin closest to the ligated artery and which had received the retrograde injection. In later experiments the injection was made antegrade, and both sutures were tied after the injection. In these instances the distribution of the infarct corresponded to that of the injected branch. More recently we have recognized that the preparatory passage of two sutures through the mesentery, by mechanical stimulation, caused the artery to undergo spasm, and this was responsible for a number of injection failures. Therefore, in recent experiments the injections have been made antegrade without any preparation; then, with the needle still in the artery, two Atraumatic silk sutures were passed, the needle removed, and the sutures tied quickly. The resulting brief hemorrhage has never proved serious. The advantage of this technique is that it has virtually eliminated injection failures. In earlier experiments a guinea pig never received more than a single serum injection, although not infrequently it had more than one arterial branch tied, as a consequence of attempted, but unsuccessful, injections. With the newer and more reliable technique, we have not hesitated to make three or four serum injections into a single animal. In all instances of multiple ligations, at least two patent arterial branches have separated the injected arteries to assure adequate collateral circulation.

The injection volume was usually 1 cc., introduced over an interval of 45 to 75 seconds. Blanching of the intestine always occurred, as a result of washing out of the blood. It was not possible to keep the rate of injection constant; so the degree and extent of blanching varied during the injection. When the injection rate was rapid, and blanching consequently pronounced, the clear fluid would partly or completely fill the mesenteric vein draining the area. Intestinal spasm and post-injection congestion were frequently noted with potent serums, but also with control materials which did not cause infarcts. The portion of

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Department of Pathology, St. Francis Hospital.

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small intestine chosen for injection did not influence the results. A few animals died overnight, but most were killed after 24 hours.

Five serums (S) were studied. All were prepared in rabbits. S1 was a commercial lyophilized anti-sheep-erythrocyte serum, reconstituted in distilled water. S2 was prepared by intravenous injection of 1 cc. of washed, packed sheep erythrocytes, followed by five weekly 2 cc. doses and by 1 cc. weekly doses thereafter. S3 was prepared by intravenous injection of 1 cc. of washed, packed sheep erythrocytes, followed by four weekly 2 cc. doses. S4 was prepared by intravenous injections of 1 cc., followed by five weekly 2 cc. doses of a guinea-pig-kidney extract: after perfusion with saline to remove blood, the two kidneys were extracted with 20 cc. of saline by treatment in a Waring Blendor for several minutes, followed by centrifugation and filtration; the extract gave a negative benzidine test for occult blood. All the above serums were considered Forssman antisera, since the Forssman antigen is present in guinea-pig tissues, as well as sheep erythrocytes. S5 was prepared by intravenous injection of 1 cc. of washed packed guinea-pig erythrocytes, followed by five weekly 2 cc. doses; guinea-pig erythrocytes do not contain the Forssman antigen. Bleedings were done 1 week after the last injection.

The serums were inactivated for 30 minutes at 56 C before use. The titers are indicated in the accompanying Table. All dilutions were made with saline.

### Results

Guinea pigs killed 24 hours after injections of potent Forssman antisera showed hemorrhagic infarcts of the small intestine. There was sometimes a small amount of serosanguineous fluid in the peritoneum. The loops of small intestine which had been exteriorized were often adherent but were readily separated to reveal the infarcted zone. The infarcts varied from 1 to 8 cm. in length. Injections in all parts of the small intestine gave the same results except that the distribution of the infarct was influenced by the anatomical distribution of the injected artery; hence the largest infarcts followed injection into arteries supplying long segments of intestine.

Undiluted and some diluted serums (1 cc.) caused complete infarcts with fairly

Comparative Potency of Serums\*

Serum	Results of Intra-Arterial Injection						Agglutination Titer	
	Intestinal Infarct		Focal Necrosis		No Lesions		Sheep RBC	Guinea Pig RBC
	Dilution	Vol., Ce.	Dilution	Vol., Ce.	Dilution	Vol., Ce.		
S1	10	0.5	100	1.0	445	1.0	0,960	Not tested
	10 *	1.0	320	1.0				
	20	1.0						
	40	1.0						
	80	1.0						
S2	1	0.6	1	0.1	10	1.0	512	128
	1 *	1.0	5	1.0	15	1.0		
	1	1.7	10	1.0	20	1.0		
	2 *	1.0	16	1.0				
	2.5	1.0						
	5	1.0						
S3	8	1.0						Less than 4
	2	1.0	32 *	1.0	64	1.0	256	
	8	1.0						
S4	16 *	1.0						16
	1	1.0			16	1.0	16	
	2	1.0						
S5	8	1.0						2,048
	1	0.1	4	0.2	8	0.5	Less than 4	
	1	0.2			10	1.0		
					16 *	0.5		
					16	1.0		
					32	1.0		
					64	1.0		
					100	1.0		

\* Each entry on the left side represents a separate intra-arterial injection in guinea pigs, and the asterisks indicate two or three trials with identical results. Each dilution represents the total volume of serum plus diluent per 1 vol. of serum; hence the numeral 1 indicates undiluted serum.



Fig. 1.—Hemorrhagic infarct of guinea-pig small intestine produced by intra-arterial injection of Forssman antiserum.

sharp borders in 25 guinea pigs (Fig. 1). The infarcted area was swollen, with dark reddish-purple color on surface and cut section. The lumen contained blood. Microscopic sections (Fig. 2) showed that the wall was thickened, due to extensive interstitial hemorrhage and vascular congestion in all layers, especially in the submucosa. The mucosa was necrotic and ulcerated, and covered by colonies of bacteria. The mucosa was infiltrated by polymorphonuclear leukocytes, which were especially numerous near areas where the bottoms of the crypts and the muscularis mucosa were preserved. The smooth muscle fibers of the muscularis were

separated by extravasated blood or by empty spaces (edema?). Their staining affinities were usually normal, but were sometimes slightly or severely reduced. The serosa sometimes showed small amount of fibrino-purulent exudate. The mesentery showed inflammatory cells, vascular congestion, and occasionally fresh thrombi in veins.

Potent serums in very small volumes (0.1-0.3 cc.) and some of the diluted serums (in 1 cc. volumes) caused less severe changes. There were nine instances of poorly demarcated zones of patchy congestion or purplish-red discoloration. Microscopic sections showed moderate vascular congestion and hemorrhage in mucosa and submucosa (much less in muscularis), dilatation of the lacteals at the tips of the villi, dilatation of the bottoms of the crypts, crypt abscesses, focal ulceration, and focal mucosal necrosis with leukocytic infiltration. Similar changes were seen at the margins of well-developed infarcts. Submucosal congestion and hemorrhage were the only changes observed with minimal doses of serum.

In four guinea pigs intussusception occurred. In at least two of these, the intus-

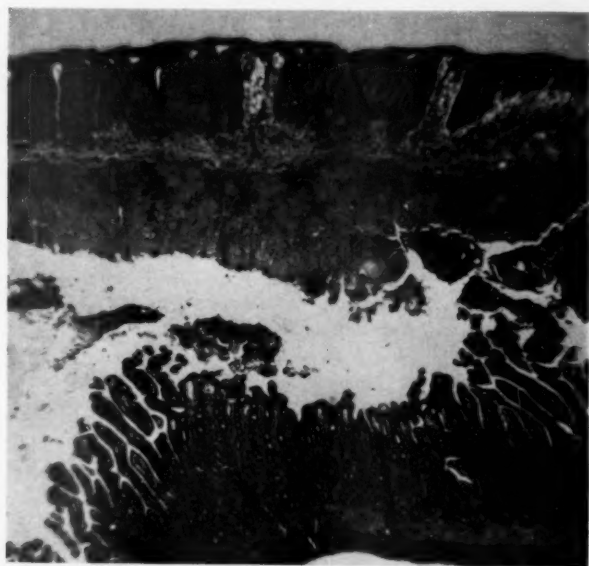


Fig. 2.—Section at edge of infarct showing necrosis, ulceration, congestion, and hemorrhage. Hematoxylin and eosin;  $\times 25$ .

susceptions were clearly unrelated to the infarcts, which were a few centimeters away. It was thought that the intussusceptions were due to the tearing and weakening of the mesentery produced by the hemostatic sutures.

*Controls.*—Simple ligation of single arterial branches (with accompanying veins) never caused infarcts, even when the distal tie was so poorly placed as to impinge on the terminal arterial arcade. Ligation of multiple branches (up to four), each separated by one or two intact branches, never caused infarcts. The following undiluted serums failed to cause infarcts: 1 cc. of normal rabbit serum, 1.7 cc. of rabbit anti-human-globulin (Coombs') serum, 1 cc. of horse serum (tetanus antitoxin), and 1 cc. of human VDRL-reactive serum. Distilled water, saline, and histamine phosphate (0.1 mg. base), each in 1 cc. volume, failed to cause infarcts, although the distilled water caused particularly severe intestinal spasm and blanching and the histamine resulted in blanching, followed by severe congestion. A few bubbles of air have occasionally been injected inadvertently with Forssman antisera diluted beyond their effective range, and in one animal 0.3 cc. of air was injected alone. Negative results were obtained in these animals, showing that air did not cause the infarcts, even though the bubbles were seen to impede the circulation temporarily.

The potent serum S1 (1 cc.) failed to produce an infarct in a single rabbit (the rabbit's tissues do not contain Forssman antigen). The potent serum S2 (1.5 cc.) was found to produce an infarct in a single kitten (the cat's tissues contain Forssman antigen). The same serum failed to produce an infarct in the guinea pig after removal of Forssman antibodies by absorption with sheep erythrocytes and guinea-pig kidney.

Injection of a potent serum into a mesenteric vein was without effect.

*Comparative Potency of Serums.*—The potency of the serums as anti-Forssman agents was assayed (after inactivation) by

test-tube agglutination of sheep erythrocytes (Table). The results indicated that S1 had high, S2 and S3 intermediate, and S4 low, titers. S5, which was an anti-guinea-pig-erythrocyte serum, had no activity, since guinea-pig erythrocytes lack Forssman antigen.

Four of the serums were tested against guinea-pig erythrocytes, and, naturally, S5 had the highest titer. The presence of activity against guinea-pig erythrocytes in one of the anti-sheep-erythrocyte serums (S2) was attributed to prolonged immunization, with consequent loss of specificity. The presence of activity against guinea-pig erythrocytes in the anti-guinea-pig-kidney serum (S4) was a surprise because the kidney had been perfused to remove blood, and the occult blood test had been negative; possibly enough erythrocytes persisted in the kidney to evoke a low titer of antibodies.

When assayed according to ability to induce intestinal infarcts (Table), S1 appeared strongest and caused infarcts in high dilutions (1:80). S2, S3, and S4 were of less, and approximately equal, strength. It was impossible to assay S5 completely because it proved very lethal. There was pronounced *in vivo* agglutination of mesenteric venous blood during intra-arterial injections of S5 even in dilutions up to 1:8, and this was responsible for a number of fatalities. Since the 1:16 and higher dilutions were inactive, it is certain that S5 was not stronger than S2, S3, and S4. It may have been weaker, but it certainly had at least some ability to produce infarcts, since injections of 0.1 cc. of undiluted S5 was effective.

*Rate of Injection.*—One cubic centimeter portions of potent undiluted serum S2 were injected very rapidly (19 seconds) in one guinea pig and very slowly (8 minutes) in another. Infarcts resulted in both animals, and they did not differ from infarcts produced by the standard 45-75-second injections. (This does not rule out the possibility that rate of injection may be important when diluted serums of borderline potency are employed. In fact, Forssman<sup>2</sup> found the rate of injection very important, but

this was at least partly due to the anatomic situation, which allowed of the possibility that a rapid retrograde carotid injection would carry the serum into the aortic arch and thus by-pass the vertebral artery and the brain.)

### Comment

The results have shown that the intestinal infarcts produced by intra-arterial injections of Forssman antisera were due to the antibodies in such sera combining with Forssman antigen in the tissues. Nonspecific effects from the ligation or from the serum were ruled out by the numerous negative controls, which included several types of immune serum unrelated to the Forssman antigen. Furthermore, the Forssman antisera were active after considerable dilution, and the activity was removed by absorption with sheep erythrocytes and guinea-pig kidney. The negative result in a rabbit and the positive result in a kitten were accounted for by the respective absence and presence of Forssman antigen in the tissues of these two species. The fact that sera prepared with either sheep erythrocytes or guinea-pig kidney were effective was strong evidence that Forssman antibody was involved.

In order to provide further conclusive evidence for the Forssman specificity of the reaction, we attempted to compare the potency of several sera by serial dilutions in the animal and in the test tube. This attempt was successful, since the serum (S1) which had the highest titer against sheep erythrocytes was also the most potent in producing infarcts. However, the anti-sheep-erythrocyte sera S2 and S3 had appreciably higher titers against sheep erythrocytes than did the anti-guinea-pig-kidney serum S4; yet all were of approximately equal potency in producing infarcts. The lack of close correlation between toxicity and anti-sheep-erythrocyte titers has been noted and explained by Taniguchi,<sup>3</sup> who showed that rabbit antisera to sheep erythrocytes contained both Forssman (heterophil) antibodies and specific

anti-sheep-cell (isophil) antibodies. The latter, although devoid of toxic action, would contribute to the test-tube titration, and thus S2 and S3 had higher titers than S4 but no greater potency in producing infarcts. It is also possible that kidney and intestine have antigens in common other than the Forssman which would contribute to the infarct-producing ability of an anti-guinea-pig-kidney serum, but we have no evidence on this point.

Forssman<sup>2</sup> observed that it was possible to reproduce the "carotid syndrome" by injections of particulate matter like lycopodium or starch. We observed that the serum S2 agglutinated not only homologous (sheep) erythrocytes but also heterologous erythrocytes (guinea pig, cat), which lack Forssman antigen. This raised the possibility that the intestinal infarcts were produced not by a Forssman mechanism but by intravascular hemagglutination and consequent embolism. However, it was soon observed that infarcts were produced by sera S3 and S4 (and their dilutions) which had no, or very slight, agglutinating activity on guinea-pig erythrocytes. The anti-guinea-pig-erythrocyte serum (S5) was prepared to add more evidence on this point. Undiluted, and in small volume, it caused visible intravascular agglutination and an infarct. But when diluted 1:16, so that its titer against guinea-pig erythrocytes was the same as the titer of undiluted S2, it caused neither visible intravascular agglutination nor an infarct. Since S2 could produce infarcts even after 1:8 dilution and partial damage even after 1:16 dilution, it is apparent that its hemagglutinating properties were of no importance. Therefore, it may be concluded that infarcts were produced by a purely Forssman mechanism (S3 and S4) or by a purely hemagglutinating mechanism (S5), and that in the case of the serum which showed both activities (S2) the hemagglutinating effect was minor; but a very small participating role has not been ruled out.

It has been shown by fluorescent antibody techniques that the Forssman antigen occurs

in vascular endothelium.<sup>4</sup> Hence it is possible that the mechanism of infarct production involves endothelial damage with altered permeability and is probably analogous to the tissue damage produced in the skin by local injection,<sup>5</sup> in the lungs by intravenous injection<sup>5</sup> ("reverse anaphylaxis," "heterophil shock"), in the brain by carotid-artery injection,<sup>6</sup> and in the pancreas by pancreaticoduodenal-artery injection.<sup>7</sup> Venous thrombi have been observed only in a few of our animals, and it is difficult to assign them great importance because they may be the result, rather than the cause, of the infarction.

Grégoire and Couvelaire<sup>8</sup> have produced intestinal infarcts in dogs by repeated intramural injections of horse serum (Arthus phenomenon). Using the same immunologic technique, it was found that infarcts could be prevented by extirpation of the vegetative nervous system,<sup>9</sup> by intravenous procaine,<sup>10</sup> and by cortisone (but not by desoxycorticosterone).<sup>11</sup> None of the procedures has been applied to infarcts produced by Forssman antiserum; so the relation between the two methods of producing infarction remains obscure.

There is little direct evidence, at present, to suggest an immunologic mechanism for intestinal infarction in humans. Nevertheless, the experiments reported here have application to clinical medicine because they show that intestinal infarction can be produced without vascular occlusion. Recently one of us (S. L.) performed an autopsy on a 64-year-old woman who died of a massive infarction involving the entire small intestine and part of the ascending colon. The superior mesenteric artery and vein and the aorta were opened *in situ*, and neither occlusions nor vascular disease was found. In addition, the heart and lungs were normal, and so there was no possible source of embolus. Similar experiences have been recorded by others.<sup>12-17</sup> Other, presumably similar cases (but without autopsy confirmation) have been reported<sup>8,18</sup> as "functional infarcts," "unexplained infarcts," or "enteritis necroticans." These reports have

been based on observations at laparotomy of infarcts which responded favorably to intramuscular epinephrine (1 mg.), to intravenous procaine (1%, 15-20 cc.), or even to general anesthesia alone, so that recovery ensued without benefit of intestinal resection. The pathogenic mechanism of these human cases may be neurogenic rather than immunologic, as Reilly et al.<sup>19</sup> have produced intestinal infarcts in animals by injections of various toxins and chemicals in the vicinity of the splanchnic nerve. Indeed, Grégoire and Couvelaire<sup>8</sup> and Longo et al.<sup>9,18</sup> consider that irritation of the autonomic nervous system is the common denominator linking their immunologic experiments with the work of Reilly et al.<sup>19</sup> Although the exact mechanism is still in doubt, the experimental evidence and clinical experience indicate that intestinal infarcts occur in the absence of vascular occlusion.

### Summary

Hemorrhagic infarcts of the small intestine of guinea pigs were produced by injections of Forssman antisera into the mesenteric arteries. Some diluted sera produced less severe damage, with patchy congestion, hemorrhage, ulceration, and necrosis. It was shown that these lesions were due to the reaction of Forssman antibodies with tissue Forssman antigen. A hemagglutinating serum was also capable of causing infarction, but hemagglutination was not a significant factor in the action of Forssman antisera.

Clinical and autopsy experience supported by experimental evidence indicates that intestinal infarcts occur in humans in the absence of vascular occlusion.

Dr. Lucie Adelsberger gave valuable advice and encouragement. Ruben Gruenewald assisted in some experiments. Cappel Laboratories donated serum S1.

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# Proteinuria Associated with Experimentally Produced Abscesses and Fever in Dogs

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Mild transient proteinuria associated with a variety of clinical and experimental febrile diseases has been recognized for many years. The nature of the proteinuria has not been well characterized, but as long ago as 1929 Spence suggested that globulins alone in the urine indicate a "transient condition."<sup>1</sup> The proteinuria has been ascribed to poorly understood lesions, such as cloudy swelling and toxic degeneration of the kidney tubules. This proteinuria may be due to an increase in the amount of filtrable protein in the serum. Such an increase in a single protein fraction of the serum may cause increased filtration, particularly if the fraction contains small molecules, such as are known to be present in the  $\alpha$ -globulin fraction. Increases of the serum  $\alpha$ -globulin fraction of patients with a wide variety of tissue-destructive lesions have been reported.<sup>2</sup> The work of Lockey et al.<sup>3</sup> shows that a component of the alpha fraction, the serum mucoprotein, is elevated in patients with neoplasms, and that such an elevation is associated with an increased amount of mucoprotein in the urine. Similar increases in serum  $\alpha$ -globulins have been observed in dogs poisoned with mercury<sup>4</sup> and in rats subjected to scalding or to turpentine abscesses.<sup>5</sup> The following work was undertaken to define, if possible, the relationships between the serum and urinary proteins during periods of febrile proteinuria in dogs with sterile turpentine abscesses. In order to distinguish between

the effects of fever per se and the effects of fever plus tissue destruction, a second group of experiments were performed, in which fever was produced by exposing dogs to high environmental temperatures.

## Methods

Healthy mongrel male and female dogs were used after several days of observation. They were kept in individual metabolism cages during the experimental periods. Timed voided urines were collected at approximately 24-hour intervals. The dogs were allowed tap water and standard dry kennel rations ad lib.

**Proteins.**—Total serum protein determinations were done by the Lowry,<sup>6</sup> biuret, and micro-Kjeldahl methods. Urine protein was prepared by centrifuging, filtering, dialyzing (24 hours vs. running tap water) and lyophilizing 25-ml. aliquots of urine. The Lowry method was then applied to this dry powder.

**Electrophoresis.**—Undiluted serum and barbital solutions of dry urine powder were separated on filter paper at pH 8.6. The strips were stained with bromophenol blue and scanned with a Photovolt densitometer. The areas of the curves were determined by planimetry. Separation of the beta and gamma fractions was not sharp in many cases, and these fractions are considered as one in this report.

**Abscesses.**—Abscesses were produced between the scapulae by the subcutaneous injection of 1-2 ml. of turpentine.

**Clearances.**—Effective renal plasma flows and glomerular filtration rates were determined by the clearances of *p*-aminohippuric acid and exogenous creatinine, respectively.<sup>7</sup>

**Fever.**—Fever was induced by placing the animal in a standard clinical fever cabinet (Hypertherm) for intervals of two to nine hours.

**Sialic Acid.**—The diphenylamine color reaction<sup>8</sup> was used for both serum and urine powder. This determination was used as a check on the electrophoretic serum  $\alpha$ -globulin values. It has been shown that the seromucoids, which migrate with the  $\alpha$ -globulins, contain as much as 10% sialic

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# PROTEINURIA—ABSCESS AND FEVER

TABLE 1.—*Experimental Abscess Formation: Serum Proteins*

Dog, No.	Weight, Kg.	Sex	Duration of Abscess, Hr.	Temperature (C)	Urine Vol., Cc/Hr.	Serum Proteins Gm/100 Ml.*			
						Total	Alb.	$\alpha$	$\beta \gamma$
8	10.5	M	0	30.3	12.5	5.3	1.7(32)	1.4(26)	2.2(42)
			31	40.5	16.2	6.4	2.5(39)	2.0(31)	1.9(30)
			72	40.0	10.8	6.8	1.8(27)	1.6(24)	3.3(49)
10	9.5	F	120	39.8	13.1	6.1	2.2(36)	1.8(30)	2.1(34)
			0	39.0	10.5	5.7	2.4(42)	1.2(21)	2.1(37)
			24	39.2	22.5	5.5	1.8(33)	1.7(31)	2.0(36)
			77	40.2	56.0	5.1	1.4(27)	1.4(28)	2.3(45)
11	12.5	M	94	39.0	7.3	5.1	1.4(27)	1.6(31)	2.1(41)
			0	38.5	10.8	6.4	2.1(33)	1.8(28)	2.5(39)
			46	40.4	16.2	5.2	1.6(31)	1.6(31)	1.9(38)
			118	39.5	12.2	6.6	1.5(23)	2.1(32)	3.0(45)
			148	39.6	9.0	6.7	2.0(30)	1.8(27)	2.8(43)
12	5.5	F	191	38.6	10.1	6.6	1.7(26)	1.8(27)	3.1(47)
			0	38.5	11.2	5.8	2.5(43)	1.3(22)	2.0(35)
			47	39.6	1.5	5.8	2.2(37)	1.5(26)	2.1(37)
13	12.0	F	71	39.8	2.8	3.7	1.0(27)	1.4(37)	1.3(35)
			0	39.3	19.0	6.3	3.1(49)	1.2(19)	2.0(32)
			23	40.6	21.0	5.5	1.9(35)	1.5(27)	2.1(38)
			42	41.4	30.0	7.0	2.5(36)	2.6(37)	1.9(27)
			71	40.8	20.0	5.4	1.6(30)	2.1(39)	1.7(31)
			96	40.0	16.0	6.1	1.6(26)	2.0(39)	1.7(31)
			131	38.5	22.0	6.1	1.9(31)	2.3(38)	2.3(38)
			142	38.3	30.0	6.3	1.8(29)	1.7(27)	2.8(44)

\* Figures in parentheses refer to percentages of the total.

acid\* and that a closely related substance, neuraminic acid, which gives the same color reactions as sialic acid, is found almost exclusively in the  $\alpha$ -globulin fraction.<sup>10</sup> The sialic-acid concentrations are expressed as optical density units (O. D.) per milliliter of serum or per milligram of protein.

*Ultracentrifugation.*—Sedimentation constants for the protein soluble in 0.2 ionic strength, pH 7.4 potassium phosphate buffer were determined for one serum and one urine  $\alpha$ -globulin fraction. The determinations were made in a Spinco Model E analytical ultracentrifuge. The  $\alpha$ -globulin frac-

TABLE 2.—*Experimental Abscess Formation: Urine Proteins*

Dog, No.	Duration of Abscess, Hr.	Urine Proteins Mg/Hr.*				Clearance Ml. Serum $\times 10^{-1}$ /Hr.		
		Total	Alb.	$\alpha$	$\beta \gamma$	Alb.	$\alpha$	$\beta \gamma$
8	0	3.5	--	--	--	--	--	--
	31	2.8	0(0)	0.9 (32)	1.9 (68)	0	4.5	9.6
	72	7.4	0.44(6)	4.3 (58)	2.7 (36)	2.6	26.2	8.2
	120	10.2	0.47(5)	6.4 (63)	3.3 (32)	2.1	36.0	16.0
10	0	0.8	--	--	--	--	--	--
	24	1.9	--	--	--	--	--	--
	77	12.5	0	9.1 (73)	3.4 (27)	0.0	65.0	14.8
	94	2.4	0.26(11)	1.5 (61)	0.67(28)	1.9	9.4	3.2
11	0	2.1	0.3 (15)	0.4 (20)	1.4 (65)	1.5	2.3	5.3
	46	7.1	1.1 (15)	4.0 (56)	0.20(29)	6.0	24.0	10.8
	118	12.3	0.4 (3)	8.8 (72)	3.1 (25)	2.7	42.0	10.4
	148	6.7	0.3 (3)	4.0 (60)	2.5 (37)	1.3	22.0	8.9
	191	12.7	3.3 (26)	3.4 (27)	6.0 (47)	19.0	19.0	19.0
12	0	1.5	0 (0)	0.40(28)	1.1 (72)	0	3.1	5.5
	47	2.1	0.1 (6)	1.5 (70)	0.5 (24)	0.5	10.0	2.4
	71	2.8	0.3 (11)	1.9 (68)	0.6 (21)	3.3	14.0	4.6
13	0	4.3	1.3 (30)	1.3 (30)	1.7 (40)	4.2	11.0	8.5
	23	2.5	--	--	--	--	--	--
	42	16.9	2.0 (12)	8.1 (51)	6.3 (37)	8.0	33.0	33.0
	71	10.9	1.5 (14)	6.2 (57)	3.2 (29)	9.5	29.5	18.5
	96	15.7	1.3 (8)	8.9 (57)	5.5 (35)	8.1	45.0	22.0
	121	14.7	0.0 (0)	7.1 (48)	7.6 (52)	0	37.0	33.0
	142	7.8	--	--	--	--	--	--

\* Figures in parentheses refer to percentages of the total.

tions were prepared by electrophoretic separation in starch blocks.

### Results Abscess

The dogs reacted to the subcutaneous turpentine with fever, anorexia, and listlessness. A fluctuant, tender abscess developed within 24-48 hours, which either ruptured or was incised, with prompt remission of the symptoms. Illustrative results of the findings in five dogs are given in Tables 1 and 2.

**Temperature.**—Although there was considerable variation in basal rectal temperatures, all showed distinct elevations (1.2 to 2.3 degrees [C]) following the turpentine injection.

**Urine Volume.**—In four of five dogs the urine volume rose roughly in proportion to the degree of fever.

**Serum Proteins.**—Total proteins showed no consistent change. The albumin fraction decreased in four of five dogs. The  $\alpha$ -globulin fraction showed a relatively sharp and consistent elevation within 48 hours. The increase ranged from 0.2-1.4 gm. %, or from 4% to 18% of the initial concentration. The  $\beta$ - and the  $\gamma$ -globulin fraction showed a less constant tendency to increase with the development of the abscess.

**Urine Proteins.**—Urine protein excretion during control periods ranged from 0.8 to 4.3 mg. per hour. The protein was composed of  $\beta$ - and  $\gamma$ -globulins, smaller amounts of  $\alpha$ -globulin and still smaller amounts of albumin. Following the abscess, the maximal protein excretion ranged from 2.8-16.9 mg. per hour. This protein was composed principally of  $\alpha$ -globulins, with smaller amounts of  $\beta$ - and  $\gamma$ -globulins and, as in the controls, very small amounts of albumin.

**Serum Protein Clearances.**—Clearances of the serum protein fractions were conspicuously altered during periods of proteinuria. The  $\alpha$ -globulin clearance rose sharply, with increases as great as eighteenfold. Increases in the clearance of the beta-gamma fraction and, to a less extent, the albumin fraction also occurred. Of interest was the

TABLE 3.—Clearances of p-Aminohippuric Acid and Creatinine in Urine

Dog	Control, Ml/Min.	Proteinuria, Ml/Min.	Recovery, Ml/Min.
12	GFR 15.3	94.7	--
	RPF 77.9	94.0	--
11	GFR 52.5	52.8	43.4
	RPF 191.3	236.0	181.5
10	GFR 46.9	34.6	--
	RPF 107.0	161.5	--

observation that the clearances of the various serum protein fractions usually changed in the same direction, but there were exceptions. Also, the clearances of the fractions did not show the same magnitude of increase or decrease.

**Glomerular Filtration Rate and Renal Plasma Flow.**—In Table 3 it is seen that the renal plasma flow for three dogs increased during the proteinuria period. The glomerular filtration rate increased in only one dog, which had an abnormally low control value.

**Sialic Acid.**—Sialic-acid values for the dogs with experimental abscesses are summarized in Table 4. The concentration of this material in the serum increased with the increase in the concentration of serum  $\alpha$ -globulin, and there was a definite tendency for the ratio of O. D. (sialic acid) per milligram of  $\alpha$ -globulin to increase with time.

When the O. D. (sialic acid) for urine protein is expressed as the ratio of O. D. to milligrams of  $\alpha$ -globulin, the content of sialic-acid-positive material per milligram of  $\alpha$ -globulin is found to be considerably less than that of serum  $\alpha$ -globulin.

**Ultracentrifugation.**—S20w values for the phosphate-soluble protein obtained from the

TABLE 4.—Sialic-Acid Values

Dog	Control O. D./0.2 Ml. Serum	Proteinuria O. D./0.2 Ml. Serum	Serum O. D./Mg. $\alpha$ -Globulin	Urine O. D./Mg. $\alpha$ -Globulin
8	167	300	0.060	--
10	210	260	0.910	0.158
11	180	300	0.720	0.430
12	185	290	0.940	0.504
13	235	315	1.120	0.238

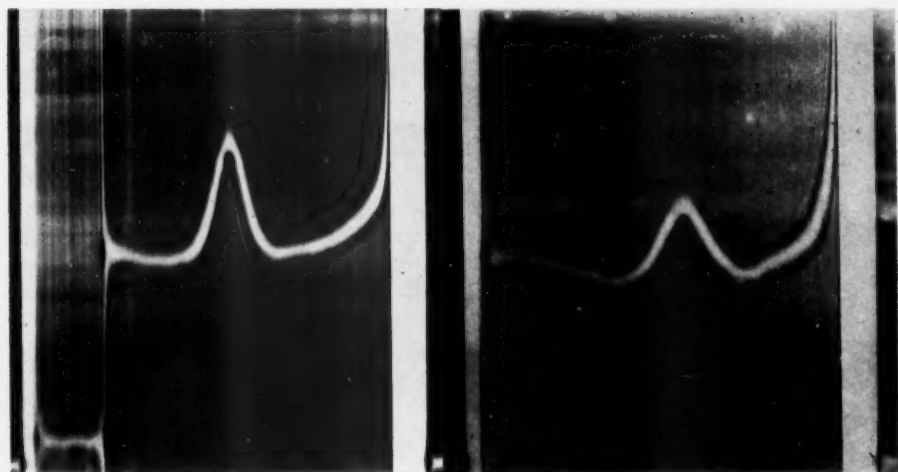


Fig. 1. (Dog 11).—Ultracentrifuge patterns for serum (left) and urine (right)  $\alpha$ -globulins. Exposures were made 61 and 129 minutes after speed (59,780 rpm) was attained.

$\alpha$ -globulin fractions of urine and serum of Dog 11 during the period of maximal proteinuria were 3.98 and 3.8 for urine and serum, respectively. Figure 1 illustrates the relative purity of these fractions. These values are comparable to those obtained for human serum mucoproteins with molecular weights of 44,000.<sup>17</sup>

### Fever

After periods of from two to nine hours in the fever cabinet, proteinuria developed in all five dogs. The findings are summarized in Table 5. Four dogs appeared clinically normal following the period of fever. One dog (No. 12) developed polyuria, anorexia hypothermia, and listlessness and died three days following the period

of fever. Autopsy disclosed no gross anatomical lesions. Microscopic sections revealed extensive proximal tubular necrosis, small areas of hemorrhage in the lungs and brain, and marked degenerative changes in the neurons of the cerebral cortex, medulla, and cerebellum, corresponding to findings reported in cases of heat stroke in the human and in experimental hyperthermia.<sup>11</sup>

**Temperature.**—During the period in the fever cabinet, with the temperature between 36.6 and 37.8 C, the rectal temperatures remain fairly constant at between 39.5 and 40.5 C. The control temperatures ranged from 38.4 to 39.2 C. The highest recorded rectal temperature was 42.0 C, for Dog 13. The cabinet temperature at that time was 40 C. Rectal temperature returned quickly

TABLE 5.—*Experimental Hyperthermia*

Dog	Sex	Wt., Kg.	Hours in Cabinet	Urinary Protein, Mg/Hr.		% Comp. of Urinary Protein *			Clearances MI/Hr. $\times 10^{-3}$		
				Control	Max.	Alb.	$\alpha$	$\beta \gamma$	Alb.	$\alpha$	$\beta \gamma$
S9	M	13.7	8	3.0	38.1	40	22	38	10.0	53.0	69.0
S12	F	4.9	9	2.6	20.7	22	31	47	42.0	64.0	51.0
S13	F	12.0	4	3.4	53.5	38	27	35	110.0	100.0	68.0
S15	F	13.0	4	4.1	22.6	17	35	48	16.0	66.0	27.0
S16	F	13.0	2	5.5	10.8	29	27	44	11.0	23.0	16.0

\* This protein analysis was obtained during maximum proteinuria.

to normal or slightly subnormal after leaving the box.

*Urine Volume.*—Urine volume increased two- to fourfold over control values during the three days following fever.

*Serum Proteins.*—Except for a transitory elevation in total protein immediately following the fever period, changes neither in total serum protein nor in the relative concentrations of the various protein fractions were noted.

*Urine Proteins.*—The excretion of urinary protein rose markedly during the first 24-96 hours following fever. The protein of the dogs with fever induced in this manner differed from that of the dogs with experimental abscesses both in quantity and in composition. After fever the dogs excreted considerably more protein, of which considerably the larger part was albumin. Clearances were correspondingly increased. The differences between the two experimental groups are illustrated in Figure 2.

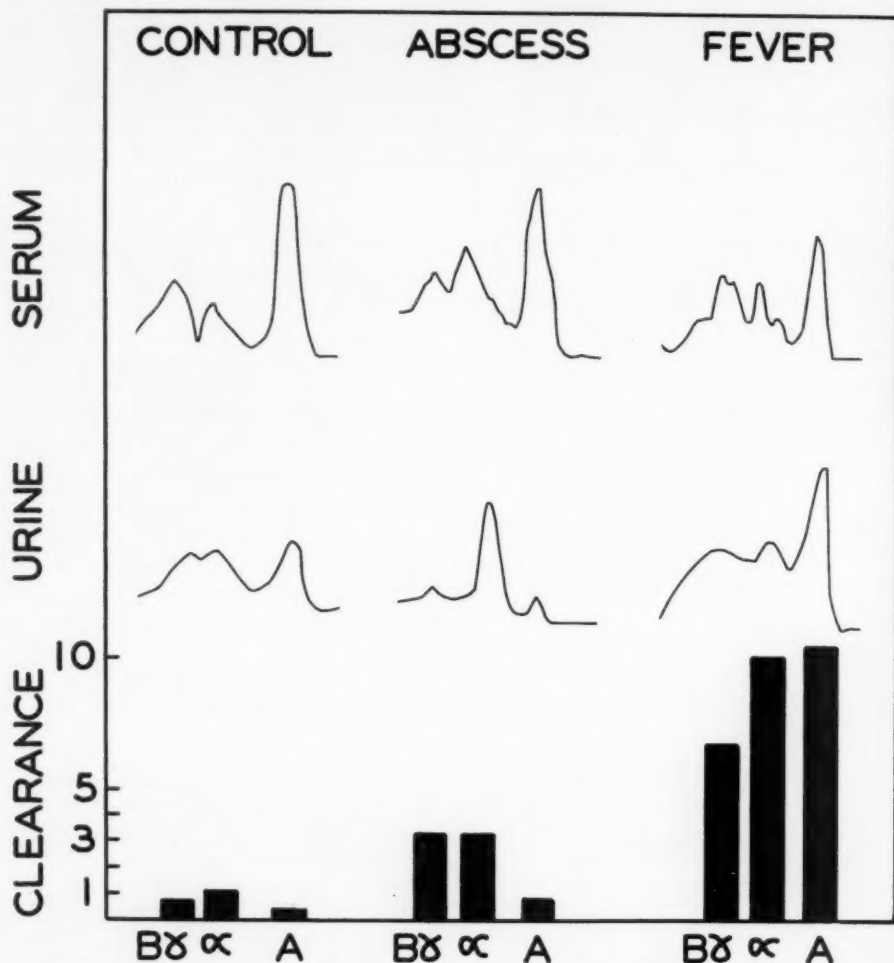


Fig. 2 (Dog. 13).—Serum and urine electrophoretic patterns for control and maximal proteinuric periods during abscess and artificial fever. Clearances (milliliters serum  $\times 10^{-2}$ ) of the serum protein fractions for these periods are also shown.

**Sialic Acid.**—No significant elevation of the sialic acid content of the serum of the dogs with induced fever was noted.

### Comment

These experiments demonstrate that with an elevation of serum  $\alpha$ -globulins,  $\alpha$ -globulinuria occurs. This finding supports the thesis that an increase in the constituents associated with the  $\alpha$ -globulins frequently found in man or experimental animals with a variety of tissue-destructive lesions may be directly related to the proteinuria commonly found. Of interest was the spontaneous occurrence of fever, elevated serum  $\alpha$ -globulins and  $\alpha$ -globulinuria in one dog not included in this study. These findings were associated with acute pyometritis and serves as a "clinical counterpart" to the experimental group. It seems reasonable to assume that in the process of abscess formation, connective tissues containing carbohydrate-protein complexes would be chemically altered and release a variety of products, including glycoproteins. Release of glycoproteins to the bath fluid has been shown to occur in dog skin thermally injured *in vitro*.<sup>12</sup>

The proteinuria does not appear to be simply a constant clearance of greater numbers of these small molecules, as evidenced by the increase in the clearance not only of the  $\alpha$ -globulins but of the  $\beta$ - $\gamma$ -globulin and albumin fractions as well. This increase might occur in several ways. There may be an increase in the number of very small molecules present in a given electrophoretic fraction which is selectively filtered and excreted: The clearance in this case would be apparently increased, although the clearance of the smaller molecules in the fraction would not. This possibility is suggested by the rising values of sialic acid per milligram of serum  $\alpha$ -globulin. Although this possible difference in the composition of the alpha fraction may explain its increased clearance, it is difficult to explain the increases in the clearances of the other fractions in the same manner. It is, however,

conceivable that the tubular reabsorption of the  $\alpha$ -globulins may inhibit reabsorption of other proteins, thus elevating their clearance rates.

An alternative possibility is that glomerular permeability is increased. Our data do not exclude this possibility, although it would seem likely that the clearance of albumin as compared with that of the beta-gamma fraction would be considerably greater than observed had the glomerular pores enlarged.

A third possibility is that there is a defect in tubular reabsorption of protein. This is given some support by the evidence of a defect in water reabsorption, indicated by the rising urine volumes and normal glomerular filtration rates. It has been suggested that tubular reabsorption of protein (measured in patients with renal disease) is nonselective, i. e., that protein molecules are reabsorbed from the glomerular filtrate strictly in proportion to their concentration in the filtrate.<sup>13</sup> This concept of the tubular epithelium as a simple membrane does not seem tenable when the kidney, whose parenchyma is largely tubular epithelium, is considered as an organ of major importance in protein metabolism, with not only reabsorptive functions but probably catabolic and synthetic functions as well. In this respect, there is some similarity to the liver. The data presented here do not fit a "non-selective" theory of reabsorption. If it is assumed that the proteins are filtered in proportion to their molecular weight, and reabsorbed according to their concentrations, one would expect urine protein to be composed of large amounts of  $\alpha$ -globulin, smaller amounts of albumin, and very small amounts of  $\beta$ - and  $\gamma$ -globulin. This is not the case in either the control or the proteinuric dogs. It appears that selective tubular reabsorption of albumin must be postulated to explain the relatively low clearance of this protein as compared with the clearance of the beta-gamma fraction. This selective reabsorption of albumin certainly must occur with respect to hemoglo-

bin. Hemoglobin is very similar to albumin in molecular size, and presumably is filtered at similar rates (or even lower rates, if present concepts of hemoglobin binding are correct), but is found in the urine at plasma levels of only approximately 100 gm/100 ml.<sup>4,15</sup>

In those animals with artificially elevated temperatures unassociated with tissue destruction, the dependence of a disproportionately large urine  $\alpha$ -globulin increase on an increase in the serum  $\alpha$ -globulin fraction was demonstrated. As in the dogs with abscesses, the data do not permit the exclusion of either increased filtration or decreased reabsorption as an explanation of the proteinuria. However, the discrepancy between what one might anticipate from increased filtration and nonselective reabsorption and the actual results indicate that selective reabsorption is one mechanism. The severe tubular damage noted in Dog S12 suggests that failure of tubular reabsorption may represent the basic defect in this type of proteinuria.

The factors operative in causing a markedly reduced half-life of albumin in the serum of abscessed dogs<sup>10</sup> may in some way affect also the renal function with respect to albumin, although our data do not bear directly on this point. Nevertheless, the accumulating evidence of the major importance of the kidney in protein metabolism should direct attention to the selective, rather than the random, functions of this organ with respect to protein.

### Summary

Proteinuria was induced in dogs by turpentine abscesses and by artificial fever. The proteinuria due to abscess formation was characterized by large amounts of low-molecular-weight  $\alpha$ -globulin. This was associated with the appearance of increased amounts of similar protein in the serum  $\alpha$ -globulin. Clearances of  $\alpha$ -globulin rose considerably, whereas the clearances of other fractions were less markedly elevated. The proteinuria due to artificial fever dif-

fers from that due to abscess formation in that more protein is excreted, and, of the total, a greater percentage is albumin. Possible mechanisms of the proteinuria are discussed, and evidence for the conclusion that selective tubular reabsorption plays a role is given.

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# Regeneration of the Fundic Mucosa in Rats

## III. Further Studies of the Effect of Corticotropin and Cortisone and of Stress

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It is generally agreed that the healing process is similar in acute and chronic ulcers of the stomach and duodenum in man. Acute ulcers heal rapidly under ordinary conditions. The best evidence shows that chronic gastric ulcer will heal in 40 days or less under strict medical management.<sup>1</sup>

Several facts strongly suggest that the adrenal cortex is closely related to peptic ulcer disease (for details see Reference 2). Prolonged administration of corticotropin or corticoids may produce fresh peptic ulcer in man or cause reactivation of a previously healed ulcer. The importance of the stress factor in the pathogenesis of gastroduodenal ulcers has also been emphasized by most reviewers on this subject, and peptic ulcer has been designated a stress disease or even an endocrine disease.<sup>3</sup>

The mechanism of the ulcer-promoting effect of these compounds and of stress is obscure. The main hypotheses in this respect are a stimulating effect on gastric secretion and an impeding effect on the formation of granulation tissue—or both—as well as interference with the protective mucous barrier.

The results reported in my previous paper<sup>4</sup> indicated that epithelization of experimental gastric defects in rats occurred at the same rate whether or not the animals were subjected to different hormonal influences. The doses of the different hormones employed were sufficient to cause typical alterations in the various endocrine glands. No clear-cut effect, however, was observed in the corticotropin-treated group, indicating that the dose was too small from

a biologic point of view (0.6 mg. per kilogram of body weight daily). The dose of cortisone was also perhaps small (1.4 mg. daily per kilogram of body weight), though some biologic effect was noted in this group.

This study is an investigation on the effect of *high* doses of corticotropin and cortisone, and of a stress factor, upon the same experimental gastric defects under otherwise identical conditions. In order to produce constant stress during the course of the experiments, the animals in this group were given injections daily of a formaldehyde solution.<sup>5,6</sup> Formaldehyde presumably elicits the general adaptation syndrome, mainly due to its local action on tissues at the site of injection. It causes no important specific organic lesions, and the drug therefore has been widely employed and has proved extremely useful even in experiments of some weeks' duration concerning the general adaptation syndrome.<sup>3</sup>

### Materials and Methods

The material and the experimental technique were the same as those fully described in my first paper.<sup>7</sup> It is specially emphasized in this connection that the suture technique in the gastric as well as the abdominal wall was exactly the same as previously, no special attention having been paid to the fact that the animals were treated with corticotropin and cortisone.

The number of animals in each group was twice as great as that previously used, each group comprising 16 rats, 8 males and 8 females, about 3 months of age. The hormone injections started one week prior to gastric operation and lasted until the animals were killed. The injections of formalin were not started prior to but on the day of operation, for fear that the animals would otherwise not survive long enough. The body weights were noted on the day that the injections were started, on the gastric operation day, and at

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## FUNDIC MUCOSA REGENERATION

autopsy. The weights of the adrenals and the pituitary glands were also noted.

Earlier experiments have shown that the 15th day after the gastric operation is best fitted for observation.

**Dosage.**—Corticotropin (Jaton Prolongatum A. L.): Corticotropin acetate was administered in daily doses of 2 mg., corresponding to 10 mg. per kilogram of body weight. The dose is about 17 times higher than the dose used formerly. Calculated according to weight only, without considering the difference in species, the dose would be 700 mg. in humans.

**Cortisone** (Cortone, Merck): Cortisone acetate was given to two groups of animals in daily doses of 2 and 4 mg., respectively. The doses correspond to 10 and 20 mg. per kilogram of body weight per day. These doses are seven and 14 times higher than the doses used before. Calculated according to weight only, the doses would be 700 and 1,400 mg. in humans.

**Formaldehyde:** Each animal was given daily injections of 0.5 ml. of a 4% solution of formaldehyde from the day of operation.

### Results

**General Effects.**—The various experimental procedures had a striking effect, which is clearly reflected in the changes in body weight of the animals and the weight of the adrenals.

The animals in the *control group* gained 8% and 9% in body weight from the start of the injections and the gastric operation, respectively, until they were killed. The average weight of the adrenals was 49 mg.

The *corticotropin-treated group* revealed a distinct adrenal hyperplasia, the adrenal weights being 35% higher than those of the control group. The body weight increased 6% and 9% from the start of injections and the gastric operation until they were killed.

The *two cortisone-treated groups* showed significantly decreased weight of the adrenals, 63% and 61%, respectively. The body weight of the highest-dosage group decreased 16% and 27% from the start of injections and the gastric operation until autopsy. The lowest-dosage group revealed a decrease of body weight of a lower degree, 13% and 19%, respectively, during the same period.

The animals in the *stress group* weighed exactly the same at autopsy as at the start of the formaldehyde injections (the day of operation). This means a lack in normal increase of weight which is even greater than it appears, owing to the fact that the body weight at autopsy included extensive edema at the sites of the formaldehyde injections. The adrenals of these animals were distinctly hyperplastic, the weight being 20% higher than in the control group. There was no statistically recognizable difference between the weight of the adrenals in the corticotropin-treated group and in the stress group, but the adrenals of both groups were significantly hyperplastic compared with the adrenals of the control group.

There was very good conformity of the values of body weights and the weight of the adrenal glands in all groups. The same relative variations were noted in both sexes of all groups except in the highest-dosage cortisone group, in which the females demonstrated a greater decrease in adrenal weights than the males.

The weight of the *pituitary glands* showed no difference between the control group and the stress group. The corticotropin-treated group disclosed 15% lower weight of the hypophysis than the control animals. The highest and the lowest cortisone-treated groups showed a decrease of 7% and 12.5%, respectively. Too much emphasis will not be laid on these values, however, and no conclusions will be drawn, because the weight may probably not be exact for these minute weights.

Four of the eighty animals died, two in the highest- and one in the lowest-dosage cortisone-treated groups and one in the stress group. Death occurred in all instances during the first day after operation, due to gastric hemorrhage or suture insufficiency in the abdominal wall.

**Influence on Gastric Defect.**—The results are shown in the Chart. Twelve of the sixteen animals (75%) in the control group displayed full epithelial covering of the defect on the 15th day. The same degree of

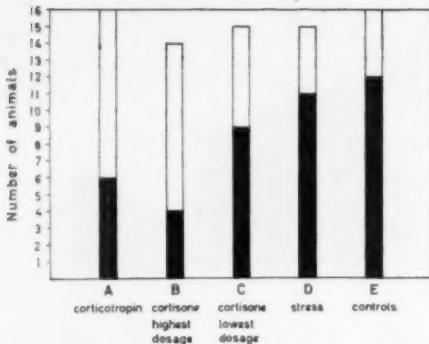
## Comment

The experimental data of the effect of corticotropin and cortisone on gastric ulcers are conflicting.<sup>8-11</sup> The majority of authors studying this problem have used doses ranging from twice up to 14 times higher than those used formerly by me.<sup>4</sup> Yet, some of the results were consistent with my first observations.

The present investigations, however, indicate that if sufficiently high doses of corticotropin and cortisone are employed these compounds can produce delayed epithelial regeneration in experimental gastric defects in rats.

Thus, the present results confirm the findings of Skoryna et al.<sup>8</sup> in thermal-induced ulcers in the rats' stomach (25 mg. of cortisone per kilogram of body weight three times weekly). It must be borne in mind, however, that the apparently retarded epithelial healing does not necessarily mean a direct effect upon epithelization per se. It is more likely that the decreased epithelial covering is merely a result of a severe effect on the connective tissue of these compounds.

The results obtained in the corticotropin-treated and cortisone-treated groups are in accordance with the generally accepted view on the effect of these hormones on the gastroduodenal tract. Very unexpected was the finding, however, that the stress group did not differ from the control group with regard to epithelial covering. This finding can hardly be explained by the fact that these animals were exposed to heavy stress only from the day of operation, while injections in the other groups started one week prior to operation. Since repeated and prolonged administration of corticotropin and adrenal steroids simulates certain forms of chronic stress in eliciting many of the same responses, including the effect on gastric function, it was expected that the severe stress on the animals in the formalin-treated group would give results comparable with those obtained in the corticotropin- and cortisone-treated groups. No convincing explanation of this finding can be given. It



Epithelial covering of defects following gastric operation in animals given corticotropin, different doses of cortisone, and formaldehyde (stress). The black columns represent the number of animals showing complete covering of the defect on the 15th day.

epithelization was observed in the formalin-treated (stress) group, in which 11 of the 15 animals (73%) showed complete covering. The cortisone-treated group with the lowest dosage revealed a tendency to delayed epithelial healing, as only 9 of the 15 animals (60%) showed complete covering on the 15th day.

A statistically significant ( $P > 99\%$ ) decrease in healing capacity was demonstrated in the highest-dosage cortisone-treated group, in which only 4 of the 14 animals (28%) showed full covering, as well as in the corticotropin-treated group, in which only 6 of the 16 rats (37%) revealed completely epithelized defects.

Diastasis was found between the muscular layers of the defect in one to three cases in all groups except the stress group, which demonstrated no diastasis.

Abscess of the gastric wall was noted in seven and eight animals of the corticotropin-treated group and the stress group, respectively, while only three and four animals in the other groups developed abscess in the gastric wall. The abscesses, however, were lying outside the wall and did not seem to influence the epithelization, which did not differ in the animals with and without abscess.

seems that the severe stress induced in these animals did not suffice to produce a hormonal effect on gastric healing, similar to that obtained by administration of corticotropin and cortisone. The stressor effect itself seemed to be severe, judged by the general effect on the animals.

This and previous articles<sup>4,7</sup> have dealt with the *epithelization* of experimental gastric defects in rats subjected to various hormonal influences. The effect on the *connective tissue*, in all series, will be the topic of the next paper.

### Summary

Further studies on the epithelization of experimental gastric defects in rats have been described. The animals were distributed into five groups of 16 animals each, 8 males and 8 females, namely: corticotropin-treated, cortisone-treated with different doses (two groups), stress (formaldehyde-treated), and a control group. Only the corticotropin-treated and the highest cortisone-dosage groups revealed retarded epithelial covering of the defects.

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# Teratoma of the Lung

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Intrathoracic teratomata are unusual, but intrapulmonary teratoma constitutes one of the rarities of medicine. Rusby,<sup>1</sup> in 1944, collected 251 examples of teratoma of the anterior mediastinum, whereas we were able to find no more than 11 documented examples of teratoma of the lung in a search stimulated by the accession of the case presently described. Moreover, the reports of intrathoracic teratoma in the older literature have been characterized by anatomic imprecisions leaving the student in doubt as to the exact anatomic relations and, indeed, site of origin.

## Literature

Mohr<sup>2</sup> is credited with reporting the first case of teratoma of the lung, in 1839. Cloetta<sup>3</sup> also reported a case of his own, occurring in the left upper lobe of a 20-year-old woman who complained of coughing up hair. The diagnosis was established at autopsy, and the author, while describing the lesion as being situated in the lung, expressed the opinion that it arose from connective tissue outside the lung proper. He further stated, with reference to Mohr's case, that it was not clear whether this latter represented a primary lesion of the lung or whether it was similar in origin to his own case. Black and Black<sup>4</sup> described a malignant teratoma of the left upper lobe treated by surgical drainage. Slaughter and Stevens<sup>5</sup> also reported, in a 33-year-old man a malignant teratoma found, at autopsy,

to occupy the greater portion of the lung. Machol<sup>6</sup> reported a cystic lesion of the left upper lobe in a 16-year-old girl who had previously been under treatment for tuberculosis. The lesion, not examined histologically but thought to represent a dermoid cyst, was found at thoracotomy to occupy the entire lobe. Goyanes<sup>7</sup> treated a teratoma of the right lung by repeated drainage and exteriorization. His 31-year-old woman presented complaints of right chest and shoulder pain, paraesthesias of the right arm, and recurrent fever and ultimately died of hemorrhage from the fungating mass. Gumenjuk<sup>8</sup> reported a 37-year-old man who coughed up hair and had a hair ball in the larynx. While the lesion was referred to as being in the lung, the evidence presented does not exclude a mediastinal location. Laffitte<sup>9</sup> described a 21-year-old woman presenting with chest and left arm pain, cough, hemoptysis, and hair in the sputum. The cyst, situated in the left upper lobe, was emptied surgically and the bronchus closed off at its point of communication. Gürkan<sup>10</sup> documented a case of a 27-year-old woman characterized clinically by fever, chest pain, and, finally, pneumothorax. The lesion was located on the right side, between the middle and lower lobes, and was treated by excision. Hauber and Asang<sup>11</sup> reported two-stage pneumonectomy for a teratoma of the left upper lobe of a 37-year-old man. Ruland<sup>12</sup> reported a malignant teratoma of the left lung also attached to many other intrathoracic structures.

## Report of Case

### History

A 46-year-old Negro woman was admitted to the University Hospital on April 22, 1958, because of hemoptysis. She had been subject to recurrent

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From the Departments of Pathology (Dr. Collier, Dr. Dowling, and Mr. Plott) and Radiology (Dr. Schneider), University of Alabama Medical Center.



Fig. 1.—Posteroanterior roentgenogram of chest; air-containing cyst.

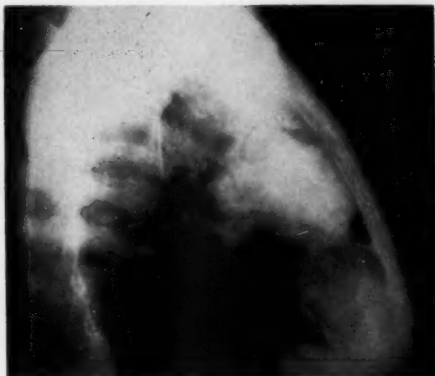


Fig. 2.—Lateral chest roentgenogram; suggested intrapulmonary location.

hemoptysis for 15 to 20 years. The expectorated blood was described as bright red, and the attacks had increased in severity in recent years. Hemoptysis had reappeared four weeks prior to admission and persisted up to the time of thoracotomy. Blood loss from this bout, the severest that she had experienced, required replacement transfusion.

Between the episodes the patient had been relatively asymptomatic but had a mild chronic productive cough, increased susceptibility to upper respiratory infections, and slight dyspnea on exertion. An attack of pneumonia in her early teens was the only significant finding in her past medical history.

The patient appeared chronically ill and ashen and was mildly febrile. Expansion of the left hemithorax was restricted, with dullness and diminished vocal fremitus in the left midlung field posteriorly. The sputum contained moderate amounts of dark red blood. Blood pressure was 240/140.

Packed cell volume after 2 units of blood was 36, but other routine laboratory studies, including prothrombin activity, were normal at this time.

Two days after admission, bronchoscopy revealed a considerable amount of fresh blood coming from the left upper lobe orifice. At the same operative session a left supraclavicular node biopsy was also performed. Neither the bronchial aspirate nor supraclavicular lymph nodes contained tumor.

#### Radiologic Findings

An ovoid, air-containing mass, measuring 9 cm. in greatest diameter, was present in the antero-medial left upper chest (Fig. 1). The mass was surrounded by air-containing lung on all sides except for its medial and inferior margins. The medial margin blended with the mediastinum and displaced the left main bronchus and lower trachea

slightly toward the right. The inferior margin was limited by focal minimal thickening of the major fissure (Fig. 2).

Within the cystic mass was a large solid ball, filling the greater part of the cavity and appearing to lie almost free within the cavity.

Review of previous films, made available to the University Hospital, revealed no interval change in the lesion over a three-year period. The patient had been aware of abnormal radiographic chest findings for at least 10 years.

Radiologic differential diagnoses included (1) dermoid cyst communicating with a bronchus, (2) bronchogenic cyst, (3) bronchial adenoma with obstruction and secondary formation of an acquired cyst.

#### Treatment

Two and a half hours after left upper lobectomy, a left hemothorax necessitated secondary thoracotomy, at which time a small bronchial vein and various areas of denuded pleura were ligatured. Blood, 3,500 cc., had been administered during the initial surgical procedure, and 2 units of serum albumin was administered after the second thoracotomy. Clot retraction was virtually absent in blood drawn at this time, and lysis of the clot was observed. A subsequent specimen of blood, however, showed partial clot retraction without lysis, a Lee and White coagulation time of less than 5 minutes, 100% prothrombin activity, and diminished platelets in the smear. There was slow improvement in clot retractability and stability following a total of 5 units of albumin and one unit of fresh blood drawn in a plastic bag to preserve platelet activity. Twenty-six hours postoperatively, however, supraventricular tachycardia developed, later progressing to ventricular fibrillation and death.

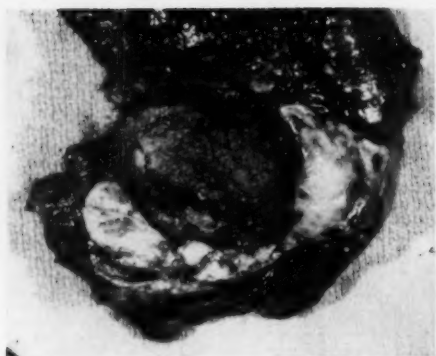


Fig. 3.—Trichosebaceous mass in situ (gross photograph).

#### Pathologic Findings

The specimen consisted of the upper lobe of the left lung, measuring  $17 \times 10 \times 6$  cm. and weighing 260 gm. (Fig. 3). A cystic structure measuring 7 cm. in greatest dimension was present in the lobe, immediately underlying the pleura. The cyst was filled with dirty-brown grumous material mixed with hair. The pale gray lining was composed of skin, which, at one point, was thrown into a number of irregular folds accentuated by a long polypoid structure. The posterior segmental bronchus communicated with the cyst cavity (Fig. 4). The cut surface of the polypoid structure consisted of fibroadipose tissue. Focal calcification was apparent. The remainder of the lobe showed moderately dilated bronchi and fibrous pleural adhesions.

Microscopic examination of the cyst disclosed a lining of a stratified squamous



Fig. 4.—Point of communication of bronchus with hairy, polypoid wall of evacuated cyst (gross photograph).

epithelium, underlying which were numerous skin appendages, including sebaceous glands, hair follicles, and eccrine and apocrine sweat glands (Fig. 5). This was also the structure of the polypoid finger-like projection described grossly. In addition, the classically observed clusters of pancreatic acinar tissue were present, but insular elements were not found (Fig. 6). The epithelial lining of the cyst was continuous with the lining of the posterior segmental bronchus, which was also stratified squamous in character, showing a moderate amount of growth activity. Proximally the epithelium merged into a more standard respiratory type. At the

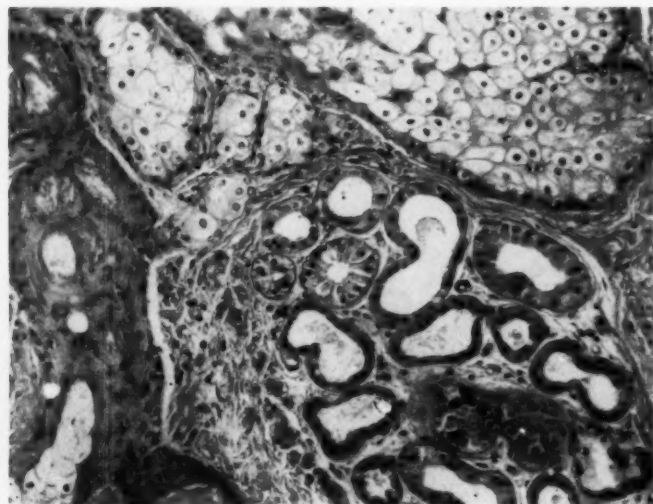
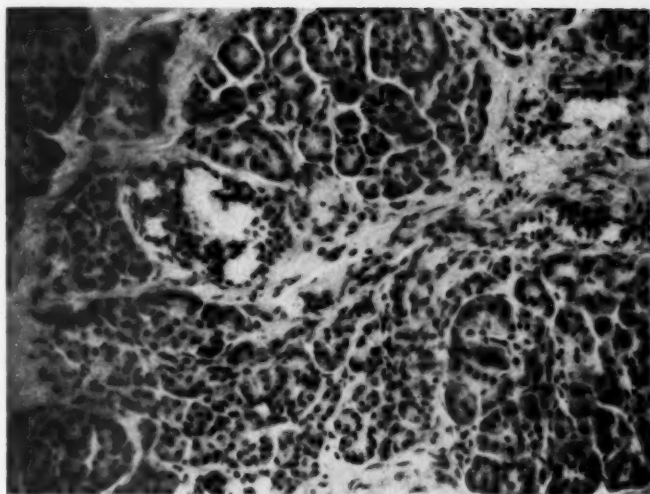


Fig. 5.—Microscopic appearance of dermal appendages in wall of cyst. Hematoxylin and eosin; reduced about 25% from mag.  $\times 430$ .

Fig. 6.—Microscopic appearance of intramural pancreatic acini. Hematoxylin and eosin; reduced about 25% from mag.  $\times 430$ .



junction of the bronchus with the cyst, the epithelium was ulcerated and necrotic and a number of medium-sized vascular channels were eroded. The adjacent lung showed hemorrhage and large phagocytes in the alveoli, while other areas of parenchyma gave the picture of organized pneumonitis. The bronchi were moderately dilated and their walls scarred. The diagnosis was benign cystic teratoma.

#### Comment

More than a nosologic nicety, accurate establishment of the anatomic location of intrathoracic teratomata is reflected in the approach to adequate surgical management of the given case. Unfortunately, however, pulmonary teratomata present with symptomatology and physical findings similar to the commoner teratomata of the anterior mediastinum. Chief among these are cough, streaky hemoptysis, pain in the chest which may radiate to the arm, signs of pressure on thoracic contents (blood vessels, bronchi, esophagus, recurrent laryngeal nerve, or sympathetic chain), and fistula formation with or without infection in the cyst. Since most of the lesions are benign, development and progression of symptoms are slow, but complications before the advent of modern thoracic surgery have been grave.

Benign teratomata are mostly of the "dermoid cyst" type, that is, consisting largely of ectodermal derivatives, such as skin, hair, and other dermal appendages. In contrast to "dermoid cysts" of ovarian origin, certain components of entodermal origin are also frequently found, e. g., pancreatic acinar tissue and structures formed of respiratory epithelium. In some cases all three germ layers may be represented. Interestingly, ectodermal derivatives are infrequently found in malignant teratomata.

The similarities of structure and symptomatology make it most likely that teratomata of lung and mediastinum have a common genesis. The most satisfying theory of development is that advanced by Schlumberger,<sup>13</sup> who postulates origin from the third pharyngeal pouch which is the anlage of the thymus. The third pharyngeal pouch is entodermal (compare the high incidence of entodermal derivatives, such as pancreatic tissue and respiratory epithelium, in these teratomata), invested by mesenchyme but also in close contact with the cervical sinus, which is part of the ectodermal third branchial cleft. Thus, tissues from any of the three fundamental germ layers may be represented in these teratomata. In support of this thesis, it is

pointed out that teratomata of the mediastinum always occur in the vicinity of the thymus and frequently are in intimate contact with this gland. In 6 of the 12 cases of teratoma of lung collected here, the lesion was situated in the left upper lobe, again implying proximity to the thymus, perhaps with migration of the anlage along the course of the lung bud. In one of the remaining cases, the lesion was situated between the right middle and lower lobes. Data on the other five are incomplete. We have no immediate explanation for this apparent predilection for the left upper lobe.

### Summary

A case of benign teratoma arising in the left upper lobe of lung of a 46-year-old Negro woman is recorded. The rarity of the pulmonary location of this lesion is emphasized.

The anlage of the thymus, as advanced by Schlumberger, is suggested as the most reasonable explanation of the genesis of these teratomata and of teratomata of the anterior mediastinum.

Eleven other cases from the literature are reviewed.

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# Atherosclerosis in Pigeons

## *Its Spontaneous Occurrence and Resemblance to Human Atherosclerosis*

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This report deals with our finding of spontaneous atherosclerosis in certain breeds of pigeons. The disorder is nearly identical with the disease in human beings. Almost no atherosclerosis was found in other breeds of pigeons kept in some instances under the same conditions of diet, housing, and exercise. The incidence and morphologic appearance of the disease in pigeons are presented in this paper.

Our interest in the pigeon is an outgrowth of experimental work using chickens.<sup>1</sup> It is difficult to induce chickens to take a measured amount of exercise by a method adaptable to large experiments. We turned our attention to pigeons because it was thought they could be exercised in reproducible fashion, using their homing instinct. Realizing the difficulty of accurately determining the sex of newly hatched pigeons, we began with autosexing pigeons, a breed in which all birds of a given sex have the same color and pattern.

### Materials and Methods

Four breeds of pigeons were used. Autosexing Kings (10 males, 10 females), Silver Kings (12

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From the Vivarium (Dr. Clarkson) and Departments of Pathology (Dr. Prichard), Neurology (Dr. Netsky), and Biochemistry (Dr. Lofland), Bowman Gray School of Medicine of Wake Forest College and the Laboratories of the North Carolina Baptist Hospital.

males, 12 females), White Carneau (12 males, 12 females), and Show Racers (6 males, 6 females) were obtained from the Palmetto Pigeon Plant in Sumter, S. C. An additional group of actively flying Racing Homers (5 males, 5 females) were obtained from three Racing Homer lofts in Thomasville, N. C.

The background of the breeds of pigeons used for the study is pertinent to this report. The Autosexing Kings were developed by the Palmetto Pigeon Plant in about 1940. The autosexing birds were produced by repeated back-crossings, with use of Silver Kings and Blue Homers as the basic breeds. The White Carneau are a highly inbred strain. The entire population of White Carneau at the Palmetto Pigeon Plant was derived from 4 pairs obtained in 1915. After a few outside matings and additions completed by 1916, these birds were inbred for 42 years. The Silver King was derived from the Silver Maltese, Silver Runt, Silver Mondain, and Silver Homer. Racing Homers were developed about 150 years ago from several breeds of pigeons not closely related to the other breeds mentioned. Show Racers are Racing Homers bred for appearance rather than racing ability. They are thus the same strain and breed but are bred for different purposes and kept under different conditions of activity.

The birds used in this study were fed a free choice of four grains; yellow corn, wheat, peas, and kafir (milo). The relative consumption of the grains was yellow corn 20% to 35%, wheat 25% to 30%, peas 20%, and kafir 15% to 35%. The pigeons received no other food with the exception of a mineral mixture. Pigeons do not consume food of animal origin. Details of diets and feeding methods are given by Levi.<sup>2</sup> Housing was identical for all birds. They were housed in pairs and engaged in active reproduction after sexual maturity.

The pigeons studied were culled because of a drop in productivity. These birds were otherwise normal but did not meet the reproductive standards of the Palmetto Pigeon Plant. This plant requires that the pigeons produce a minimum of 12 squabs per year, a very high figure. The ages of the pigeons ranged from 4 to 8 years. Critical data dealing with life expectancy of the pigeon are

lacking. Levi<sup>3</sup> states that the average reproductive age limit of 6 years for the pigeon might be compared to "middle age" in man. Pigeons have been known to live as long as 30 years.

The birds were killed in our laboratory with intravenous pentobarbital sodium. The heart, aorta, and brain were removed at necropsy. Some of the aortas were stained grossly with Sudan IV by the method of Holman<sup>4</sup>; the remainder were studied without macroscopic staining. The organs were fixed in 10% neutral formalin for histologic examination.

The preparations were stained with Sudan IV and hematoxylin and eosin. In addition, sections were prepared by the method of Verhoeff for elastic tissue, Mallory for connective tissue, von Kossa for calcium, Shultz for cholesterol, and Rhinehart for acid mucopolysaccharides.

### Results

Approximately 10% of the intimal surface of the thoracic aorta of Autosexing Kings, Silver Kings, and White Carneau was covered by raised, yellow plaques projecting into the lumen (Fig. 1). Plaques were found in all birds in these three breeds. There was no difference between the sexes. The plaques were macroscopically sudanophilic. The lesions tended to occur more often at sites of bifurcation, but some plaques were seen along straight segments of the aorta. The arch of the aorta was not involved. Most plaques were seen at the distal end of the thoracic aorta. The surface of the plaques usually was smooth, but in some cases there was ulceration or central hemorrhage. A few plaques almost completely occluded the lumen of the vessel. There was no macroscopic evidence of lesions caused by vascular obstruction.

Atherosclerosis was almost absent in the Racing Homers and the Show Racers. One Show Racer had a slight amount of atherosclerosis in the aorta, but all others were free from plaques.

The microscopic features of the atherosclerotic lesions are nearly identical with those in the human being. There was an accumulation of intracellular and extracellular neutral fat (Fig. 2), cholesterol crystals, and calcium salts in the intima, separated from the lumen by a layer of fibrous connective tissue of varying thick-

ness (Figs. 3, 4, and 5). Lymphocytes and macrophages, including multinucleated forms, were abundant in some plaques but were few in others. Bone was present in the base of a few lesions (Fig. 4). Acid mucopolysaccharides were increased in the vicinity of the plaques. The internal elastic membrane was disrupted in the larger plaques, with distortion of the adjacent media (Fig. 6). There were abundant accumulations of collagen in the media near the intimal plaques. A thrombus was attached to an ulcerated plaque in one bird. Granulocytes were seen in the media and adventitia near plaques in a few cases.

Atherosclerosis of the coronary arteries was found in one heart; a coronary artery contained an atherosclerotic plaque occluding approximately one-third of the lumen. Atherosclerosis of the cerebral arteries was not seen. One brain contained an old cerebellar infarct, but the cause was not determined.

### Comment

Of the species commonly used as laboratory animals, spontaneous arteriosclerosis has been reported as occurring in the dog,<sup>4</sup> cat,<sup>5</sup> rabbit,<sup>6</sup> rat,<sup>7</sup> and chicken.<sup>8</sup> Atherosclerosis is said to occur spontaneously in the baboon.<sup>9</sup> Spontaneous arteriosclerosis of many animals housed in a zoo has been described by Fox.<sup>10</sup> He reported the incidence of spontaneous arteriosclerosis among pigeons at the Philadelphia Zoological Gardens as being 0.4%. The morphologic characteristics of the disease were not given.

Most spontaneous arterial lesions of animals are predominantly fibrous. The plaques in the pigeons are strikingly similar to the human lesion, lacking only the complications of thrombosis and total occlusion. The number of pigeons used may be too small to include such phenomena, for these secondary effects are not found in a high percentage of comparable human material.

It is of interest that almost all the lesions were in the aorta; a single plaque was found in a coronary artery but none in the brain. This is similar to the probable mode of

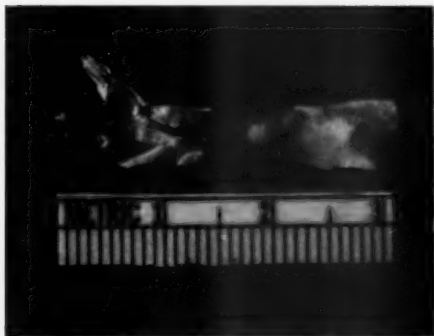


Fig. 1.—Photograph of inner surface of thoracic aorta, including the origin of the abdominal aorta. Three chrome-yellow plaques project into the lumen near the abdominal aorta. Reduced about 25% from mag.  $\times 2.5$ .

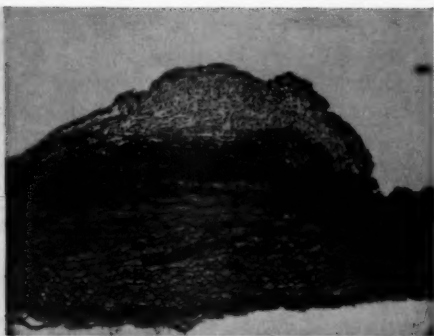


Fig. 2.—Photomicrograph of elevated plaque on inner surface of aorta. There are masses of free fat in the depth of the plaque and smaller amounts in the media. Proliferated connective tissue overlies the dark sudanophilic material. Sudan IV; reduced about 25% from mag.  $\times 85$ .

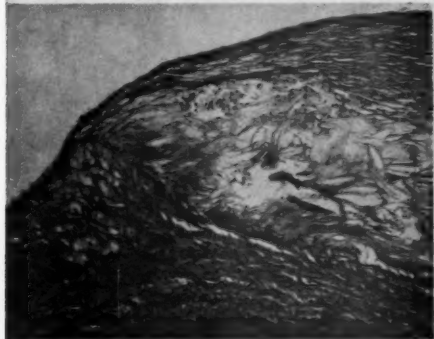


Fig. 3.—Photomicrograph of aortic plaque with cholesterol-crystal clefts and overlying proliferated connective tissue. The cellular reaction is minimal. Hematoxylin and eosin; reduced about 25% from mag.  $\times 85$ .



Fig. 4.—Photomicrograph of aortic plaque showing clefts left by cholesterol crystals, lymphocytes, macrophages, and multinucleated cells. A spicule of bone is seen in the lower right-hand corner. Hematoxylin and eosin; reduced about 25% from mag.  $\times 85$ .



Fig. 5.—Photomicrograph of small aortic plaque composed of proliferated connective tissue on the intimal surface. Free fat was seen in this plaque with use of the Sudan IV technique. The media is normal. Hematoxylin and eosin; reduced about 25% from mag.  $\times 85$ .

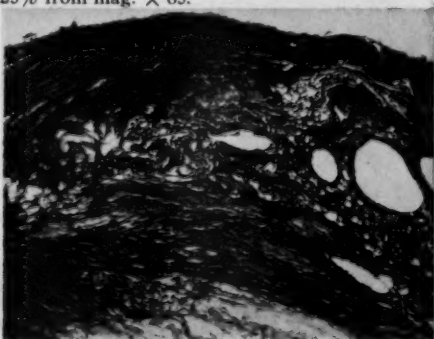
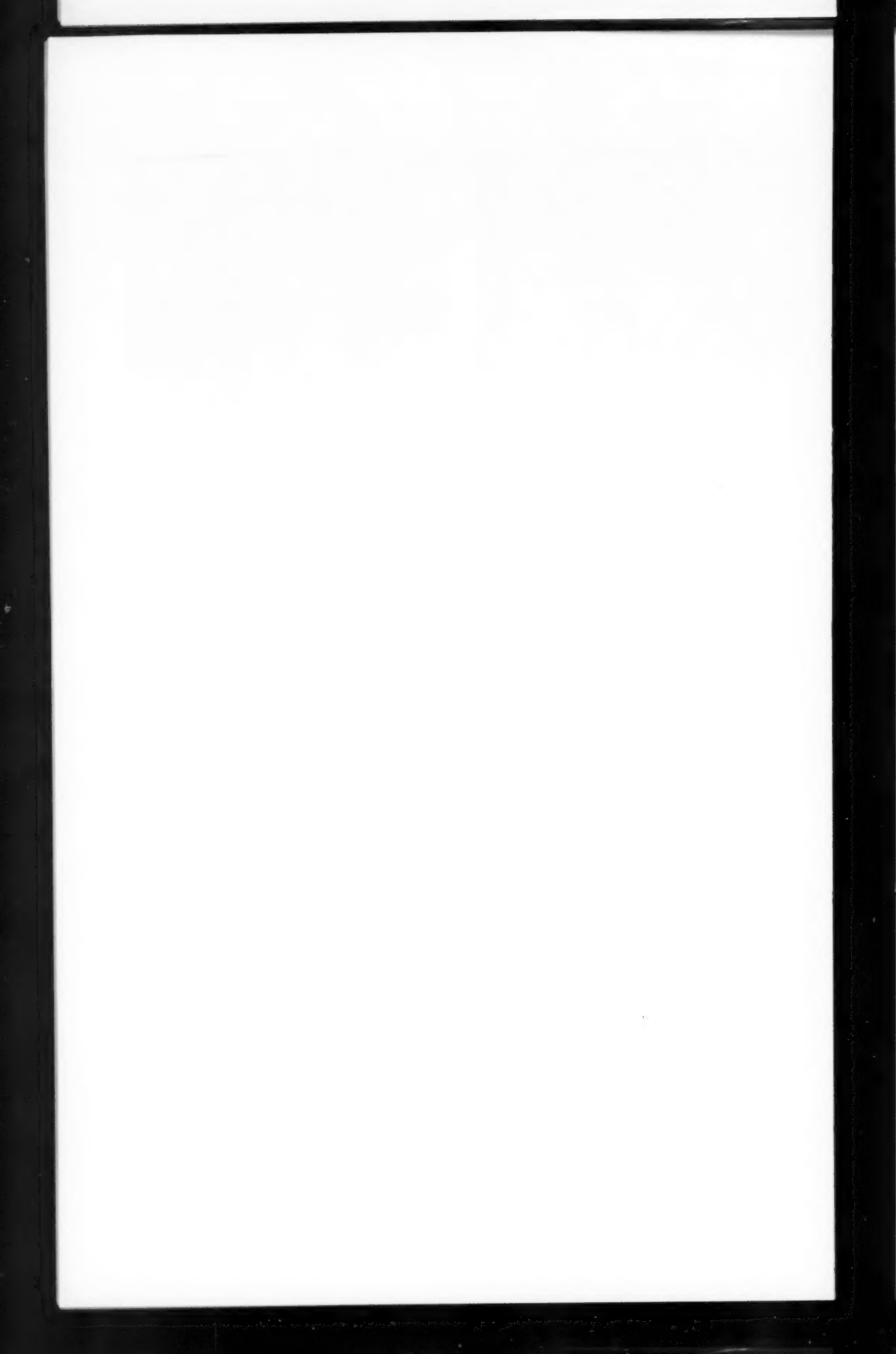


Fig. 6.—Photomicrograph of an aortic plaque compressing and distorting the media. Only a few elastic fibers remain, staining dark in this preparation. Verhoeff's elastic tissue stain; reduced about 25% from mag.  $\times 85$ .



progression of the disease in man.<sup>11</sup> Another comparable feature of the disease in pigeons and people is the sparing of the aortic arch.

The diet of the pigeons consisted entirely of vegetable material, as contrasted with at least some of the other species in which spontaneous atherosclerosis has been reported. The oils in the diet included those occurring naturally in corn and the other grains, but a protective effect was not found.

The absence of lesions in two breeds of Homers suggests genetic factors as the cause of resistance of the breeds to the disease. This is further emphasized by the similar findings in Show Racers, kept at a low level of activity, and the Racing Homers, which were active flyers. There were no sex differences in any of the breeds.

The atherosclerotic pigeon affords many possibilities for future research. The Palmetto Pigeon Plant each year culls about 5,000 pigeons aged 5 to 6 years. On the basis of our material, it may be predicted that almost all of these birds will be in good health, with atherosclerotic plaques occupying about 10% of the aortic surface. The birds have a 42-year pedigree and are kept under rigidly controlled conditions of housing and diet. Pigeons are not omnivorous, but a reasonable range of feeding experiments could be tried. These breeds of pigeons do not have significant homing ability, but they could probably be trained to fly home and thus could have measured amounts of vigorous exercise.

Biochemical studies are being done on this material. They are designed to study the relationships between blood lipids, the arterial lipids, and the severity of atherosclerosis. These studies have been reported.<sup>12</sup>

### Summary

Pigeons of the Autosexing King, Silver King, and White Carneau breeds have spon-

taneous atherosclerosis closely resembling the human disease, grossly and microscopically. Pigeons of different breeds, the Show Racer and Racing Homer, have almost no atherosclerosis. The evidence suggests that atherosclerosis in the pigeon is not related to sex, diet, or exercise but rather to a genetic factor and age.

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# Electron Microscopy in Experimental Hypertension

## *Glomerular Changes in Choline-Deficiency-Induced Hypertension in the Rat*

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When weanling rats are subjected to a period of choline deficiency, acute renal tubular<sup>1</sup> and glomerular<sup>2</sup> injury occur. Over a period of several months following the episode of dietary choline deficiency hypertension develops gradually,<sup>3,4</sup> eventuating in severe chronic hypertension. The morphological evidence of tubular injury disappears rapidly after the acute injury, but a progressive thickening of the glomerular capillary wall then occurs. This sequence of changes has been studied with light microscopy, with use of the periodic acid-Schiff stain.<sup>5</sup> Since the glomerular lesion is constant and is the only significant renal lesion observed in these animals during the development of hyper-

tension, the nature of this lesion as revealed by electron microscopy was studied.

### Methods

Weanling piebald rats of the Long-Evans strain and albino rats were placed on a choline-deficient diet for a period of two weeks and then returned to a normal diet. Kidneys, obtained by killing the animals or by unilateral nephrectomy, were studied at 8 to 12 days after beginning the choline-deficient diet, at 3 to 6 months after the choline-deficient period, and at 1 year or later, when chronic hypertension was well established.

Blocks of kidney tissue were obtained for paraffin sections and for electron microscopy within one minute after death of the animal or after nephrectomy. Tissue for light microscopy was fixed in cold neutral buffered formalin; that for electron microscopy, in an osmium-dichromate solution (Dalton's fixative).<sup>6</sup> The tissue for electron microscopy was dehydrated in alcohol, embedded in methacrylate, sectioned at 0.025 $\mu$  thickness with use of a diamond knife<sup>7</sup> and a Porter-Blum microtome. The sections were studied with an RCA-EML

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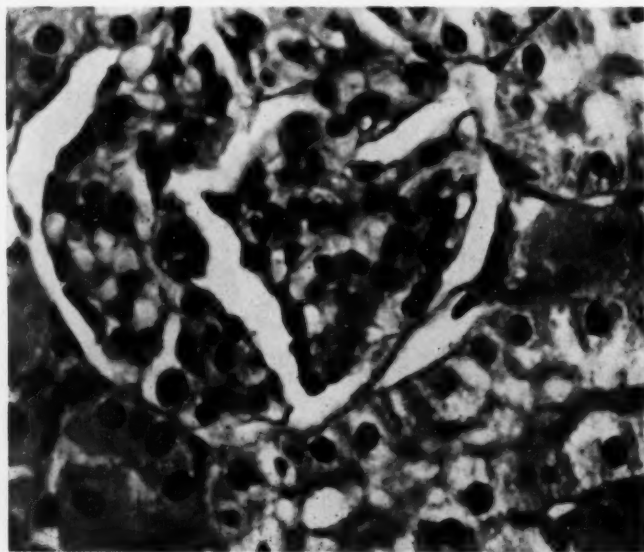
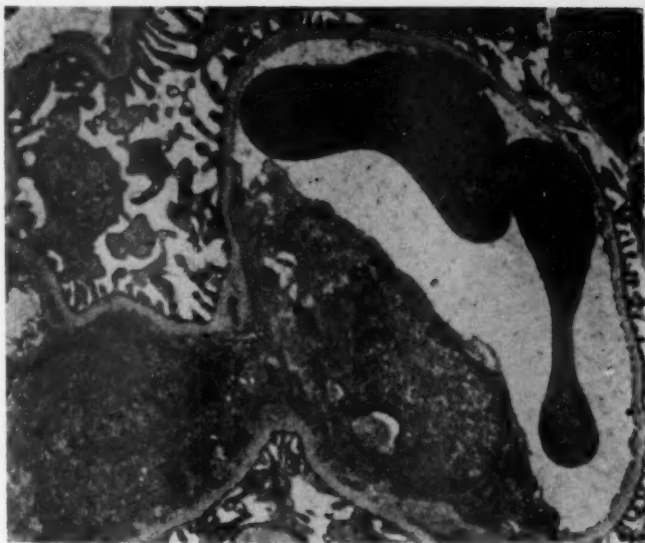


Fig. 1.—Kidney of weanling rat immediately after period of choline deficiency. The capillary walls in the glomerulus are slightly thickened. This is probably due to swelling of the surrounding epithelial cells rather than to thickening of the basement membrane. Swollen, vacuolated cells are noted in some of the adjacent proximal convoluted tubules. Hematoxylin and eosin;  $\times 880$ .

Fig. 2.—Normal glomerulus of rat. A glomerular capillary is shown, containing two red blood cells. An endothelial-cell nucleus is present, and the thin, perforated endothelial lining is visible. The delicate trabeculae of the epithelial cells and wide, open intertrabecular spaces are shown. At this magnification the slit pores are almost invisible;  $\times 500$ .



microscope. The paraffin sections were stained for fat with oil red O, hematoxylin and eosin, and periodic acid-Schiff (PAS) stains.

### Observations

Evidence of acute tubular injury was found in paraffin sections at the 8th to 12th days of the period of choline deficiency. Focal fatty degeneration, cloudy swelling, vacuolation, and necrosis of the tubules were observed, but in many instances there was evidence of recent autolysis and liquefaction of the dead tubular cells. In these acute stages, the glomeruli appeared relatively bloodless and the capillary walls were slightly thickened, as seen with hematoxylin and eosin stains (Fig. 1). However, the PAS stain failed to show any evidence of thickening of the basement membrane.

At this stage of acute injury, electron microscopy revealed changes in the endothelium and epithelium of the glomerular capillary walls. These changes were apparent upon comparison with normal control rat kidneys of comparable age (Fig. 2). In the normal capillary wall the endothelial lining appears in most areas as a thin membrane of cytoplasm interrupted by regularly spaced pores of uniform size.<sup>8</sup> The cytoplasm becomes thicker, and the pores are

not apparent around or near the nuclei of the endothelial cells. In the kidneys from rats during or at the end of the period of choline deficiency, the endothelial cytoplasmic membrane was thickened in some areas and its pores were wider than normal. The basement membrane itself was not thickened.

The most notable alteration was found in the epithelial cells. The normal glomerular capillary wall presents prominent cleft-like spaces between the delicate trabeculae of the epithelial cells. These broaden on the surface of the basement membrane, forming the pedicles. Fine slits or openings (slit pores) are present between the pedicles. These are about 100 Å in width. Numerous wide, intercommunicating cavernous sinuses between thin trabeculae, through which the glomerular filtrate can flow readily, are prominent. In the kidneys of weanling rats during and immediately after the choline deficiency, the trabeculae were notably thickened and more irregular and appeared to be fused in many areas. The entire mass of the cytoplasm of the epithelial cell was increased in amount, and the intertrabecular spaces were reduced in size and number (Fig. 3). At this time also, fine, osmium-stained droplets of fat were first noted in



Fig. 3.—Glomerulus of rat immediately after a two-week period of choline deficiency. the trabeculae of the epithelial cells are swollen and the intertrabecular spaces reduced in size. A few widened slit pores are present. The endothelial membrane is slightly thickened;  $\times 5,000$ .

the proximal convoluted tubules, near the base of the cell (Fig. 4). Some of these were visible as very fine particles in fat stains, with use of light microscopy. Large pale, rounded bodies, distinctly different from mitochondria, were found in the proximal convoluted tubular epithelium (Fig. 5). These are also present in the normal kidney, in which they are fewer and smaller. They are believed to represent the PAS-positive droplets seen in normal kidney tubules but

which are increased in prominence after choline deficiency.<sup>5</sup>

In the period following the acute deficiency episode and extending to the late stages of chronic hypertension, the light-microscopic study reveals progressive thickening of the basement membrane, as shown by the PAS stain (Fig. 6). The capillary loops were gradually narrowed, while ectasia of the intraglomerular portion of the afferent arteriole had occurred. With elec-

Fig. 4.—Kidney of rat immediately after a two-week period of acute choline deficiency. A portion of the base of a proximal convoluted tubule is found on the upper portion in which osmium-stained fat droplets are present. No fat droplets are found in the distal convoluted cells in the lower part;  $\times 5,000$ .

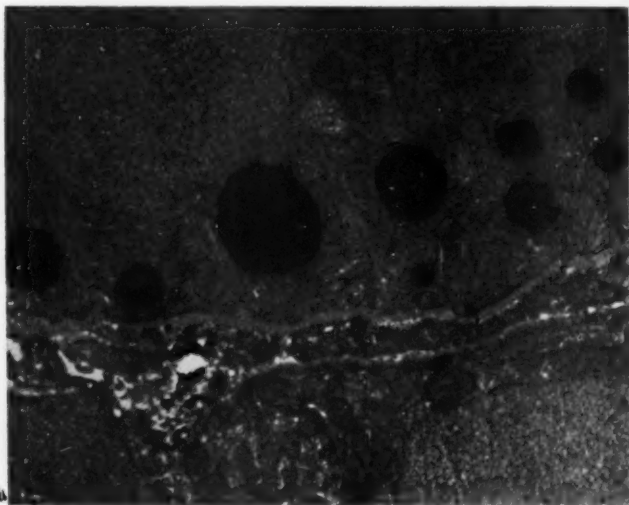
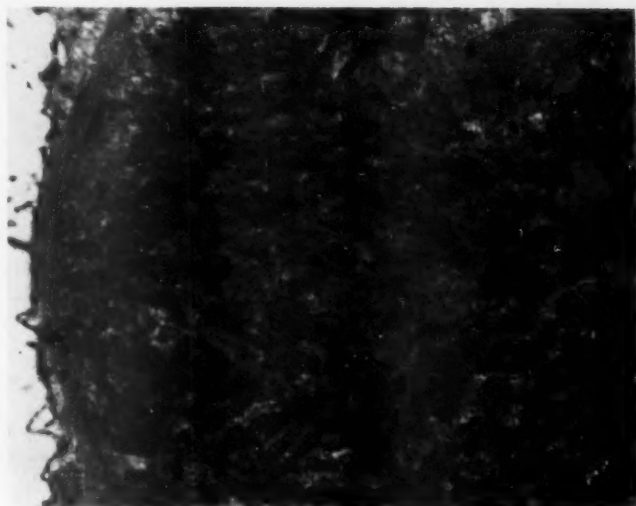


Fig. 5.—Kidney of rat three months after period of acute choline deficiency. The proximal convoluted tubule is shown. On the left is a peritubular capillary space. In the cytoplasm of the tubular epithelium are several rounded bodies of less density than mitochondria. These are the PAS-positive droplets found in the proximal tubule cells with light microscopy;  $\times 5,000$ .



tron microscopy the basement membrane was found to be irregularly thickened to as much as two or three times the normal (Fig. 7). The membrane manifests its usual homogeneous density. At this time no abnormalities can be seen in the endothelial membrane. The epithelial cells were still swollen, and their trabeculae and pedicles

were considerably thickened, and some were fused. In some areas the intertrabecular spaces were considerably reduced in size or obliterated. Some of the slit pores were of normal width. Thickening of the parietal cells of Bowman's membrane and of the parietal basement membrane could be recognized also in electron micrographs at this late stage.

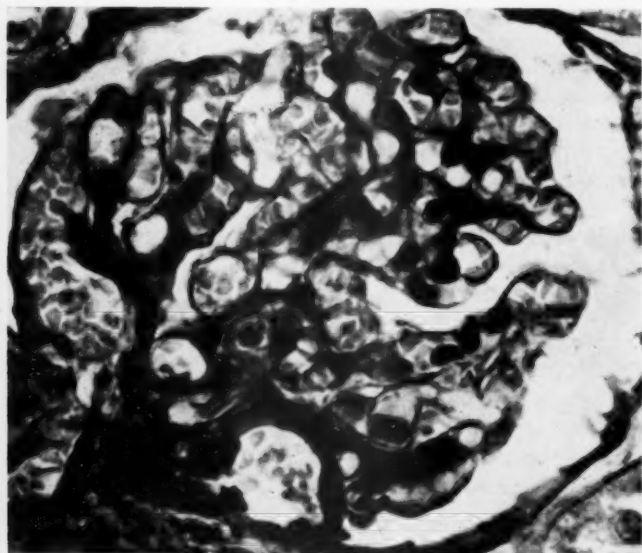


Fig. 6.—Kidney of rat one year after period of choline deficiency. Chronic hypertension was present. The afferent arteriole is thick-walled and dilated. There is noticeable thickening of the basement membrane of the capillary loops. Periodic acid-Schiff, Alcian blue, hematoxylin;  $\times 880$ .

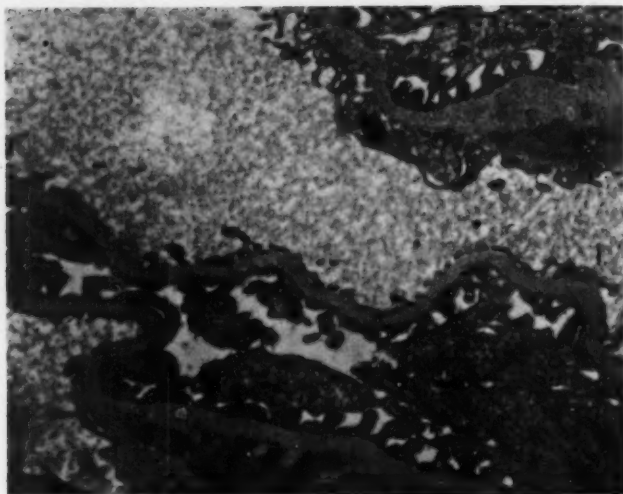


Fig. 7.—Glomerulus of rat with chronic hypertension after period of choline deficiency. The epithelial-cell trabeculae are thickened and the intertrabecular spaces narrower than normal. Several slit pores of increased size are present. The basement membrane is thickened;  $\times 5,000$ .

### Comment

It is generally held that the injury of the kidney which occurs following acute choline deficiency is due to ischemia. Fulton and Lee<sup>9</sup> have shown an increased sensitivity of blood vessels to epinephrine after choline deficiency. Prolonged vasoconstriction of the interlobular arteries, or of afferent arterioles, would be expected to cause ischemia of the glomeruli and of the proximal convoluted tubules, which are supplied by the efferent arterioles. The location and distribution of the degenerative changes in the glomeruli and tubules described in choline deficiency is in accord with the concept of an ischemic origin. Injury of glomeruli by ischemia is known to produce thickening of the glomerular capillary walls and, especially, of the basement membrane.<sup>10</sup> With electron microscopy this alteration is also apparent in rats with hypertension induced by choline deficiency. The effects upon the glomerular capillary wall are shown in this study to include swelling of the endothelial cytoplasmic lining, alteration of the pore system in the endothelium, swelling of epithelial-cell trabeculae and pedicles, reduction of the intertrabecular spaces, and slight widening of the slit pores between the epithelial pedicles. This occurs in the stage

of acute injury and persists, in some degree, as hypertension develops.

Although the morphological changes in glomeruli and tubules which follow a period of acute choline deficiency may be explained by ischemic injury, metabolic injury of the glomerular and tubular epithelial cells induced by interference with transmethylation within the cell would also explain these lesions.

The changes noted in the glomerular capillary walls would be expected to result in an increased permeability to protein, thus explaining the observed albuminuria. The thickening of the glomerular wall and reduction of the intertrabecular spaces might interfere with the normal formation of glomerular filtrate, leading to a reduction in its volume and thus producing a disturbance in the excretory function of the kidney.

The pathogenesis of the renal lesions which develop in rats following an episode of acute choline deficiency might be speculated to occur as follows: Acute choline deficiency first produces an acute injury of glomerular endothelium and epithelium and of proximal convoluted tubular cells. This could either be due to a metabolic cell injury or be the result of ischemia from sustained

## CHOLINE-DEFICIENCY HYPERTENSION

arteriolar constriction. The acute glomerular injury is followed by thickening and fusion of the trabeculae and pedicles of the glomerular epithelial cells, focal widening of the slit spaces between the pedicles, and progressive thickening of the basement membrane. As a result of this damage to the glomeruli, an increased movement of protein from the capillary through the glomerulus, an increased rate of reabsorption through the proximal convoluted tubule cells, and decreased volume of glomerular filtrate would result. These phenomena could be associated with functional disturbances in the humoral control of the blood pressure by the kidney, thus leading to hypertension.

The changes in the glomerular capillary wall associated with the development of hypertension in rats which have been made choline deficient during weaning are of an unobtrusive nature, not readily seen with light microscopy, even though present at a very early stage. Hence, the reported finding of normal glomeruli during the early stages of essential hypertension in the human<sup>11</sup> requires further evaluation with use of electron microscopy.

### Summary

The acute lesions in the renal tubules and glomeruli which occur in the rat after a period of choline deficiency at the time of weaning consist of focal fatty, parenchymatous, and vacuolar degeneration; necrosis of the proximal convoluted tubules, and swelling of the glomerular capillary walls. In the acute stage, electron microscopy reveals the glomerular lesion to consist of swelling of the endothelial cytoplasmic lining, swelling and fusion of the trabeculae and pedicles of the epithelial cells, focal widening of the slit pores between the pedicles, and reduction of the intertrabecular spaces. In the later stages, with the development of hypertension, the basement membrane of the capillary wall in the glomerulus undergoes thickening and the trabeculae and pedicles of the epithelial cells remain swollen and undergo further fusion. It is suggested that alterations in the composition of the

glomerular capillary wall may be associated with abnormalities in permeability to protein, with reduction of the volume of glomerular filtrate, and possibly may be related to a disturbance of humoral mechanisms involved in the production of hypertension.

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# Rheumatic-like Nodules Occurring in Nonrheumatic Children

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## Introduction

During the past 10 years of experience at the Children's Hospital, there have been nine children from whom subcutaneous nodules have been removed. These children had no recognizable stigmata of rheumatic disease and are considered normal and healthy at the present time. In spite of this, the nodules which were removed from the nine children, as will be described, are closely similar to or identical with rheumatic nodules. It is the purpose of this paper to review the clinical (Tables 1 and 2) and pathologic features of these cases.

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From the Department of Pathology, Children's Hospital.

## Materials and Methods

All cases at Children's Hospital having a diagnosis of rheumatic nodule, based on histologic features, were reviewed. The tissue sections were prepared with hematoxylin and eosin, Gomori's trichrome stain, Wilder's reticulum stain, and Mallory's phosphotungstic acid hematoxylin stain for fibrin.

The patients with this diagnosis received a careful history and physical examination, together with a complete blood count, urinalysis, electrocardiographic study, and one or more tests for the acute-phase reactants, including erythrocyte sedimentation rate, C-reactive protein, and serum mucoprotein-tyrosine. Antistreptolysin O titers are not available for analysis. The tests for acute-phase reactants were carried out in order to demonstrate some evidence of a systemic disorder. These procedures have been discussed by Stollerman et al.<sup>1</sup> and Adams.<sup>2</sup> Follow-up examinations were carried

TABLE 1.—*Rheumatic-like Nodules*

Case No.	Age, Yr.	Sex	History
1	2 11/12	F	The patient dropped a milk bottle on the dorsum of her left foot 3 mo. prior to admission. A small, nontender nodule appeared, growing slightly over a period of 3 mo. History otherwise negative for this healthy child.
2	2 3/12	F	"Tumor" on palmar surface of middle phalanx of left ring finger, present for approximately 1 mo., asymptomatic. History otherwise negative for this healthy child.
3	3	F	"Bumped shin" one year prior to admission. Mother noted swelling after trauma. This "tumor" persisted and became firm, nontender, and movable. Patient otherwise healthy.
4	9 6/12	M	Nodule on left temple present for 3 mo.; slowly enlarging. Patient otherwise healthy.
5	4 6/12	M	Nodule in parietal area of scalp for 9 mo., nontender and movable. At time of surgery several small satellite nodules were also palpable. Patient otherwise healthy.
6	5	F	8 1/2 mo. prior to admission "lump" developed on extensor surface of forearm just distal to elbow. Three months prior to admission it was removed at another hospital. Another mass occurred just distal to the previous site. Both are growing slowly. Second nodule removed 3 mo. after first. One month after second nodule was excised, a small red nodule appeared on anterior aspect of right ankle; growing slowly for the past 6 mo.
7	11 days	F	Nodule in parietal area of scalp noted at 1 wk. of age; excoriated because of location. Patient otherwise considered to be a healthy newborn infant.
8	7 2/12	F	One year prior to admission patient noted nodule on dorsum of left foot and on adjacent anterior portion of ankle. Several satellite nodules were also discovered. Nodules were nontender and movable. Patient otherwise healthy.
9	2 9/12	M	Four months prior to admission, mother noted painless swelling on left forearm with progressive growth. Three months prior to admission a similar nodule was noted on lateral aspect of right forearm, approximately 4 in. proximal to wrist. No history of trauma, pain, heat, or tenderness. Patient otherwise healthy.

## RHEUMATIC-LIKE NODULES

TABLE 2.—*Rheumatic-like Nodules*

Case No.	Preoperative Diagnosis	Operative Findings	Rheumatic Work-Up *	Pathological Diagnosis
1	Giant-cell tumor; tendon sheath; neurofibroma	Mass, 3.5X2 cm., attached to subcutaneum and extensor tendon sheaths	Negative	Rheumatic-like nodule
2	Lymphangioma	Nodule, 0.5X0.5 cm., on palmar fascia and flexor tendon sheath	Negative	Rheumatic-like nodule
3	Organized hematoma	1.5 cm. group of firm nodules attached to sheath of tibialis anticus; scarred subcutaneum	Negative	Rheumatic-like nodule
4	Sebaceous cyst; lipoma; fibroma	2 cm. nodule lying on temporalis fascia	Negative	Rheumatic-like nodule
5	Tumor of scalp	Nodule 0.2X0.5 cm. with smaller ones adjacent lying in subcutaneum and on galea	Negative	Rheumatic-like nodule
6	Unknown	2 cm. nodule in subcutaneum and on deep fascia; 2.5 cm. mass in subcutaneum, flexor surface of right ankle	Negative	Rheumatic-like nodule (first nodule called rheumatic nodule)
7	Exostosis; bone cyst; calcified hematoma	1 cm. nodule on galea		Rheumatic-like nodule
8	Chronic granuloma	0.5 cm. nodules in subcutaneum and on aponeurosis	Negative	Rheumatic-like nodule
9	Fibromas	2 cm. tumor, left, with a 1 cm. nodule just distal, left; 1.5 cm. nodule on right forearm on muscular fascia		Rheumatic-like nodule

\* See "Materials and Methods."

out during the following year by the attending physicians.

### Pathology

Grossly, these nodules are included in a mass of fibrous connective tissue. They are

nonencapsulated and present as firm, glistening, gray-white nodules with a pearly and coarsely granular appearance on cut section. Microscopically, the nodule is composed of a fibrovascular tissue in which there are

Fig. 1.—Hematoxylin and eosin stain; X 120.

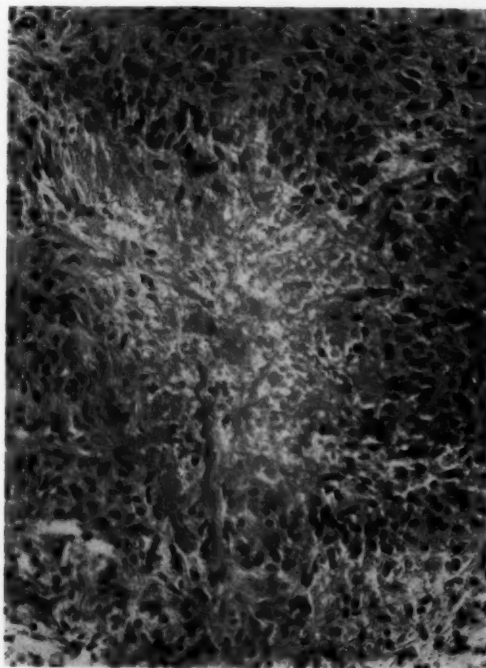




Fig. 2.—Hematoxylin and eosin stain;  
× 120.

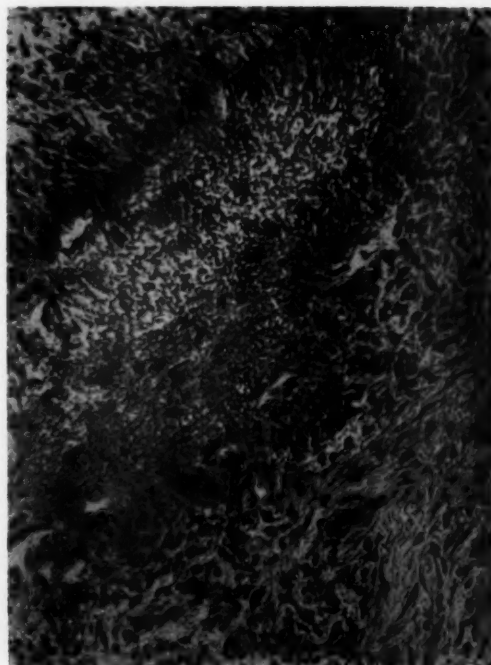
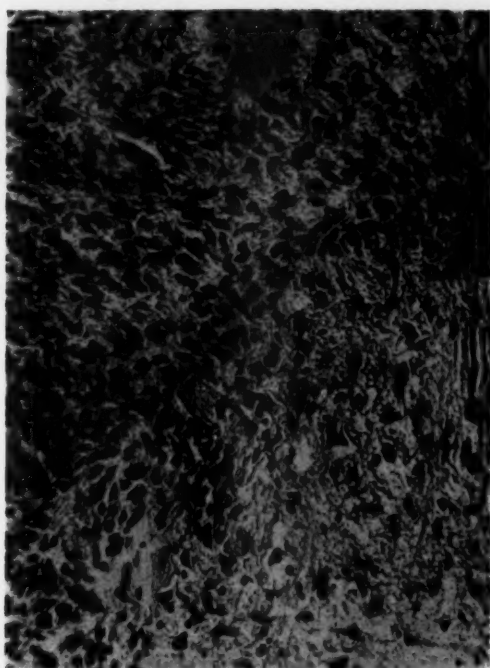


Fig. 3.—Hematoxylin and eosin stain;  
× 120.

Fig. 4.—Hematoxylin and eosin stain;  
 $\times 120$ .



scattered irregular foci of a fibrinoid or coagulation necrosis. In some areas of necrosis, ghosts of the preexisting cells may be seen. Surrounding these areas of necrosis are proliferating fibrocytes and histiocytes. These cells form a fasciculation around the necrosis. Scattered throughout the tissue are chronic inflammatory cells (Figs. 1, 2, 3, and 4).

The trichrome stain did not reveal any significant amount of collagenous connective tissue in the necrotic debris or in the adjacent proliferating cells. The fibrin stain revealed positive staining material in the necrotic areas, while the reticulum stains were positive for reticulum material throughout. The fibers appeared condensed and were generally parallel in the necrotic debris.

#### Comment

Several interesting features are noted in this group of children. First, the occurrence of rheumatic nodules in children with rheumatic disease is extremely rare in this geo-

graphic area.<sup>8</sup> Second, the children in the present group lack any of the stigmata of rheumatic disease; this is of particular interest, since it has been stated that the occurrence of rheumatic nodules is often associated with carditis.<sup>4-6</sup>

It is interesting to note that Ziegler<sup>9</sup> reported a case of rheumatic nodules occurring in a 7-year-old girl. The nodules occurred around the elbows and the left knee. They were histologically verified. They would spontaneously regress, but recurred at 11 and 13 years of age. The case was considered rheumatic in nature, although there was no evidence of joint or cardiac involvement. He considers the nodules to have been the only manifestation of rheumatic disease.

Klinge<sup>10</sup> also reports rheumatic nodules in patients without other manifestations of rheumatic disease, viz., joint or heart manifestations. The ages in his cases ranged from 5 to 73 years. It is difficult to establish whether or not these cases actually represent true rheumatic disease.

Findlay<sup>6</sup> notes that cases of "rheumatic" subcutaneous nodules without accompanying manifest signs of cardiac involvement probably belonged in a doubtful category. Keil<sup>7</sup> remarks that "clinical observation has, therefore, revealed the almost invariable association of evidence of organic valvulitis in cases exhibiting genuine rheumatic subcutaneous nodules," and the statement has also been made that rheumatic nodules are rare in children under 2½ years of age.<sup>4</sup> It would seem highly unlikely that these children are suffering from some form of rheumatic disease.

Since the histology of granuloma annulare and that of a rheumatic nodule are similar, if not identical, the possibility of rheumatic disease is considered but questioned because of the anatomic location of the nodules. It is stated by most dermatopathologists<sup>4,11</sup> that the differentiation of these two lesions is simple, because granuloma annulare occurs in the corium, while rheumatic nodules occur in the subcutaneum.

Grauer<sup>12</sup> has reported "granuloma annulare" in a subcutaneous region of the scalp. Pillsbury, Shelly, and Kligman<sup>13</sup> state that rarely are granuloma annulare lesions found *only* in the subcutaneous tissue. Recently Dannenberg et al.<sup>14</sup> have reported a case of "granuloma annulare" in the galea aponeurotica of a 4-year-old boy.

In all of our cases the nodules were in the subcutaneum or associated with deeper structures, such as fascia, tendons, or periosteum.

Because there is a history of trauma antedating several of these cases, and because a not dissimilar florid proliferation of fibrocytes and histiocytes occurs in certain forms of fasciitis, it is postulated that these cases represent an unusual reaction to trauma, not unlike proliferating fasciitis.

### Conclusion

The similarity of these lesions to rheumatic nodules is so noteworthy that it must be kept in mind when one is entertaining the possibility of the latter diagnosis. The prog-

nosis is strikingly different. In otherwise healthy children the diagnosis of rheumatic disease must be made with great caution or not at all. It would seem apropos to study these children with subcutaneous nodules to make certain that they are not suffering from rheumatic disease. Once it is certain that rheumatic disease does not exist, then consideration of this benign local reaction must be made. It is probably a matter of semantics whether one chooses to call these nodules atypical granuloma annulare lesions. The etiology is not clear in either case, although trauma may play a part in both. Tuberculosis was not a problem in our cases, however.

### Summary

Nine cases of local soft-tissue lesions resembling rheumatic nodules are described in children. None of these children had any other stigma suggesting rheumatic disease.

It is postulated that this lesion represents an unusual reaction to trauma.

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# Fatty Cirrhosis in the Rat

## I. A Method of Grading Specimens

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The evolution of a fatty liver to cirrhosis can be witnessed in rats maintained on diets deficient in choline precursors. By killing animals at intervals after the diet is begun, the transition from fatty liver to a large cirrhotic liver, and finally to a small cirrhotic liver can be demonstrated. A classification based upon four stages that are recognizable by histologic changes has been found useful as a means of grading such material. These stages can be visualized to best advantage in young, rapidly growing male rats that develop intensely fatty livers on a low-protein, high-fat diet.

### Experimental Methods

The gross and microscopic features of the liver in 287 rats that died or were killed after 1 to 26 weeks of feeding a choline-deficient diet, termed C-8 (Table 1), were analyzed. The animals were selected from a total of 487 male rats of a uniform stock\* that were started on the diet, in groups of 24 or 48, at various times during a five-year period. The rats, 35 to 42 days of age (body weight,  $110 \pm 10$  gm.), were fed the experimental diet immediately upon delivery from the supply house. This diet resulted in a severe degree of choline deficiency; 17% of the rats died of bilateral hemorrhagic renal necrosis between the second and the fifth week. The animals were individually caged. Coprophagia was minimal, since fecal pellets dropped through wide-mesh cage bottoms. The animals were allowed 8 gm. of diet per day. Food was distributed three times a week in ointment-jar feeding cups. All uneaten food was discarded on feeding days. Food consumption was not measured. Diets were prepared once each week and were

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From the Department of Medicine, University of Minnesota Medical School.

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\* Holtzman Rat Company, Madison, Wis.

TABLE 1.—*Hypolipotropic Diet: C-8*

1. Casein.....	8.0
2. Lard.....	37.95
3. Sucrose.....	48.375
4. Salts.....	4.00
5. Cystine.....	0.625
6. Vitamin powder.....	1.00
7. A DT (in oil).....	0.05
Total.....	100.00

1. Vitamin-free test casein — Gen'l. Biochem., Inc.	
2. Commercial	
3. Commercial	
4. Salt mixture No. 2, U. S. P. XIII; Gen'l. Biochem., Inc.	
5. L-cystine	
6. Vitamins, crystalline, in powdered sugar:	
Thiamine.....	0.3125
Riboflavin.....	0.5000
Pyridoxine.....	0.3125
Calcium pantothenate.....	1.2500
Nicotinic acid.....	1.2500
Menadione U. S. P.....	0.3125
Powered sugar.....	996.0625
7. Vitamins A and D plus tocopherol, in oil	
6.25000 mg. vitamin A (200,000 units/gm.-oil) *	
0.78125 mg. vitamin D (400,000 units/gm.-oil) *	
25.00000 mg. dl-Alpha tocopherol (oil) *	
17.96875 mg. peanut oil	
50.00000 mg.	

\* General Biochemicals, Inc.

refrigerated until used. Diet C-8 is a modification of that designed as LVI by György and Goldblatt in 1949.<sup>1</sup>

In the course of these experiments 67 satisfactory specimens were obtained at the time of death. Histologic specimens were not prepared from 86 rats because of postmortem autolysis. In 82 rats the diet was changed after 12 to 17 weeks to observe the disappearance of fat; these rats were not included in this study. The remaining 222 animals were killed after varying intervals on the deficient diets. They were anesthetized with ether, the abdomen opened, and the abdominal aorta severed. The median lobe of the liver was preserved intact in 10% neutral formalin in order to study the gross appearance after fixation. Longitudinal slices of the left lateral lobe were fixed in neutral formalin or in Bouin's fluid for histologic study. For fat stains, frozen sections were cut  $15\mu$  thick and stained with oil red O.

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TABLE 2.—*Stages in Fatty Liver Disease in the Rat in Severe Choline Deficiency*

	Periods, Wk. *
I. Centrolobular fat	1-2
II. Entire liver fatty	2-3
III. Uniform periportal regeneration	
A. Fibrosis absent	3-7
B. Fibrosis present (hypertrophic fatty cirrhosis)	5-14
IV. Irregular periportal regeneration	
A. Regenerative nodules (just detectable)	9-17
B. Regenerative nodules (involving one-half of liver)	12-24
C. Regenerative nodules (involving entire liver — atrophic fatty cirrhosis)	>20

\* Time periods are applicable only to the conditions of the present study; the character of the diet, the sex and strain of rat, and the age of the animal when the hypolipotropic diet is started, all influence the rate of progress.

### Observations

Four recognizable stages in the evolution of fatty cirrhosis, as seen in young and rapidly growing rats maintained in a continued state of severe choline deficiency, are described in Table 2. The appearance of the liver surface in each stage is shown in Figure 1. The macroscopic features are distinctive in Stage IV; histologic examination is required to recognize the earlier stages.

### Morphological Features

**Stage I: Centrolobular Fat.**—The accumulation of fat droplets in liver cells adjacent to the central veins represents the first histologic evidence of choline deficiency. Stage I has been designated as one in which approximately one-half of the hepatic parenchymal cells contain fat droplets (Fig. 2). In severe choline deficiency, there is no abrupt cessation of fat deposition when one-half the liver cells become fatty, as the term Stage I might suggest. The justification for the designation, i. e., a stage in which the process of fat accumulation is mainly limited to centrolobular areas, stems from the observations in male rats fed the experimental diet to which choline was added at a level of 0.2% or 0.4%. This amount of choline is inadequate to prevent some fat accumula-

tion. In such rats, the liver may remain at Stage I for as long as six months.

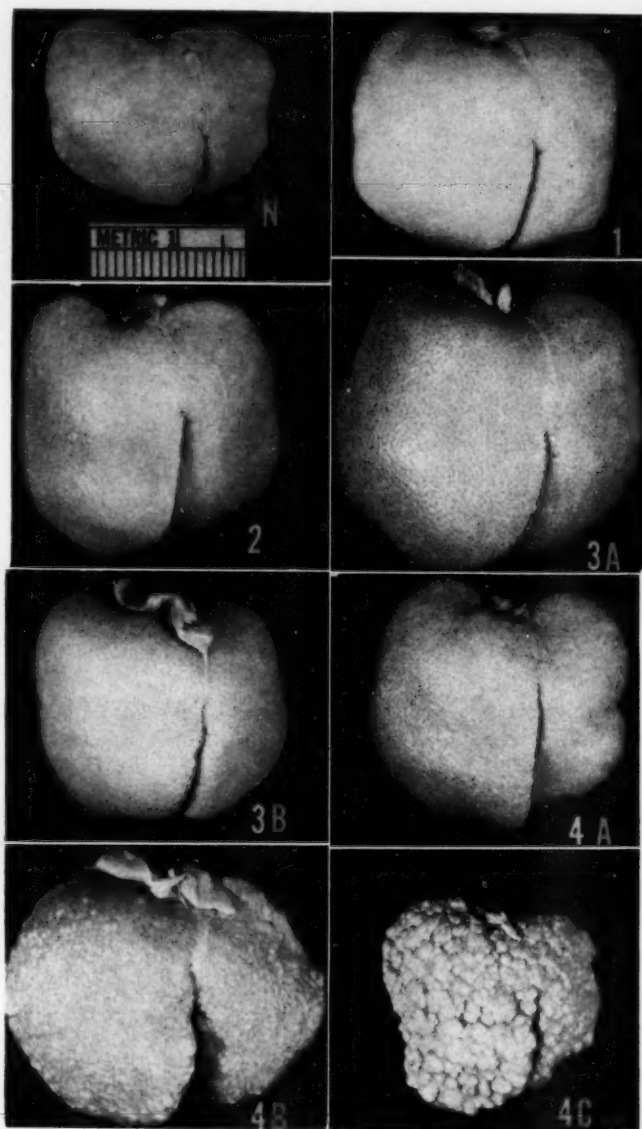
**Stage II: Entire Liver Fatty.**—In young rats maintained on diets severely deficient in choline, the entire liver becomes involved by the process of fat accumulation within one to two weeks (Stage II). Virtually every liver cell contains fat droplets (Fig. 3), and the cells in the region of the central veins are so distended with fat that they rupture. By fusion with adjacent ruptured cells, fatty cysts are formed.<sup>2</sup> At this stage the terminal portal veins are not readily apparent in tissue sections; they are obscured by the fatty cells in the immediate vicinity. The individual fat droplets within such cells are smaller than those in the hepatic cells about the central veins.

The formation of fatty cysts appears to be a mechanism by which liver cells in the choline-deficient rat are disrupted,<sup>3</sup> and probably rendered useless as functioning units. In the rat the walls of the fatty cysts appear to be formed by the remnants of many liver cells. When fatty cysts have formed in abundance, the liver has been deprived of a large number of functioning cells. This reduction in the functional reserve of the liver creates a situation analogous to that in which a partial hepatectomy has been performed in a normal rat. In each case the response is active parenchymal-cell regeneration.

**Stage III: Uniform Periportal Regeneration.**—The third stage, one in which regeneration is prominent, quickly follows the transitory second stage. Actually, some regeneration begins in Stage I, i. e., before every liver cell contains fat. Fatty liver disease is a dynamic state in which the destructive force, cellular disruption, and the response, active cell regeneration, are virtually concomitant. Nevertheless, it is convenient for descriptive purposes to consider the sequence of events as though they were static and occurred in stages.

Stage III is characterized by the presence of nonfatty cells in the vicinity of the terminal portal veins, i. e., in Zone 1 of the

Fig. 1.—The median lobe of the rat liver (after fixation in formalin) in the various stages of fatty liver disease (all photographs taken at same magnification). As fat accumulates in the liver, enlargement occurs. Active liver-cell regeneration, initially uniform throughout the liver, is an additional factor in the enlargement (Stage IIIA). In Stage IVA, regenerative nodules are just discernible as small protuberance on the surface (see also Fig. 6). Thereafter (Stages IVB and IVC) they become dominant and are conspicuous features as the final phase, an atrophic nodular cirrhosis, develops. Reproduced from article by the author published in *Minnesota Medicine* (40:603, 1957, Fig 8), by permission of the publishers.



liver acinus.† Such cells are newly formed and contain little, if any, fat. In Stage III there is a brief early phase (IIIA) in which

† The acini (structural and functional units of the liver), described by Rappaport and associates<sup>14</sup> became well delineated with the development of fatty cirrhosis, as Hartroft<sup>8</sup> has demonstrated in the rat.

fibrosis is not yet apparent. This is followed by a more prolonged phase, in which fibrosis is a relatively prominent and conspicuous feature (IIIB, hypertrophic fatty cirrhosis). Throughout this stage the formation of fatty cysts, produced by the fusion of ruptured individual fat-filled cells, continues, and the response to this loss of



Fig. 2.—Stage I of fatty liver disease in the rat. The accumulation of intracellular fat involves the areas about the central veins. The liver cells about the portal veins, particularly the terminal branches, are as yet uninvolved. Hematoxylin and eosin; reduced to 85% of mag.  $\times 50$ .

Fig. 3.—Stage II. The entire liver lobule is involved. Fatty cysts (scarcely discernible at this magnification) are beginning to form in the areas about the central veins. At this low magnification the terminal portal veins are difficult to recognize because the adjacent hepatic cells contain fat droplets. Hematoxylin and eosin; reduced to 85% of mag.  $\times 50$ .

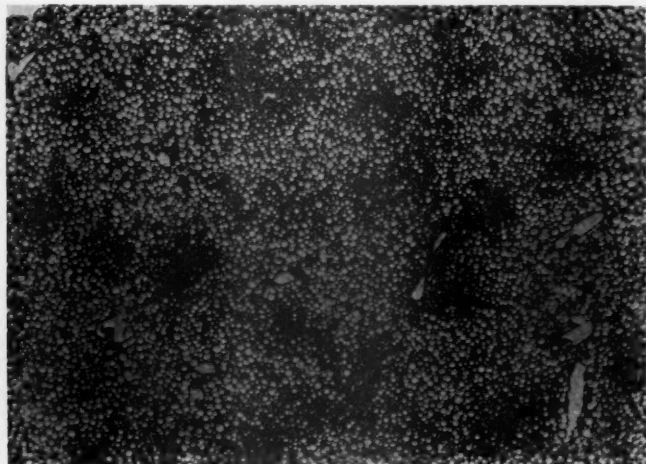
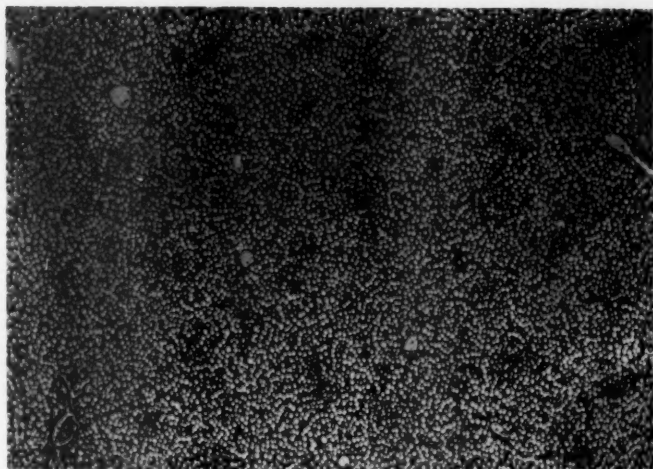


Fig. 4.—Stage IIIA. Newly formed cells appear in the areas close to the terminal branches of the portal vein. Initially, these cells are free of fat (see text). Fatty cysts, some undergoing collapse, occupy the centrolobular areas. Trabeculae will later appear in the pathways formed by the chains of fatty cysts that link central vein to central vein. Hematoxylin and eosin; reduced to 85% of mag.  $\times 50$ .

functioning cells is a continued formation of new cells. These foci of regenerative activity appear to be relatively uniform and equal in Zone 1 of each and every acinar unit throughout the entire liver.

The photomicrograph illustrating Stage IIIA (Fig. 4) demonstrates "islands" of nonfatty cells located about the terminal branches of the portal vein. The absence of visible fat droplets in the cells of the periportal areas does not constitute proof that these cells are newly formed. Fat can be rapidly mobilized from fatty cells in the periportal areas by modifying the diet. The addition of a lipotropic agent or the sudden withdrawal of all food will mobilize such fat in 24 to 36 hours. It is necessary to know the experimental conditions that have prevailed in order to interpret the significance of fat-free cells in the periportal areas. The specimen used for Figure 4 was obtained from an animal in which the food consumption was measured immediately prior to killing; neither starvation nor the addition of a lipotropic agent was involved.

In Stage III active liver-cell regeneration predominates in the areas about the terminal portal veins. Cells undergoing mitosis are seen in the periportal areas more frequently than in the areas adjacent to the central veins. The presence of fat droplets in individual cells does not prevent such cells

from undergoing mitotic division. Williams<sup>4</sup> has illustrated this in his paper, and we have repeatedly observed it in our material.

An example of Stage IIIB is shown in Figure 5. One of the characteristic microscopic features of early cirrhosis in a choline-deficient rat is the appearance of membranous septa or trabeculae that link central vein to central vein. The nonportal nature of these trabeculae has been demonstrated many times.<sup>5-8</sup> The terminal branches of the portal vein and the accompanying bile ducts and arteries are not incorporated in the membranous trabeculae. The larger portal spaces, i. e., those containing conducting branches of the vein, are incorporated in the membranous trabeculae.<sup>6</sup>

By studying sections of livers in Stage IIIB, one can observe that the pressures produced by the expanding areas of new-cell formation in Zone 1 of each liver acinus determines the pattern of the trabeculae that extend from one central vein to another. They appear to take this course because they are pushed or squeezed by areas of new-cell formation that are expanding uniformly. Initially the trabeculae contain only a few strands of collagenous tissue; gradually these become delicate sheets or membranes between the compressed tissues. In appropriate specimens obtained during Stage IIIB all degrees of the development of the



Fig. 5.—Stage IIIB. Trabeculae link central veins and large conducting portal veins. The location of these fibrous strands (membranous septa cut on edge) is determined by the pressures exerted by areas of relatively uniform liver-cell regeneration, each centered about a terminal branch of the portal vein. Virtually every individual liver acinus is thus outlined by the trabeculae. Hematoxylin and eosin; reduced to 85% of mag.  $\times 50$ .

## FATTY CIRRHOSIS IN RAT

membranous trabeculae can be witnessed. In the early phases the trabeculae are incomplete, but later they link central veins and also central veins to conducting branches of the portal vein. A relatively complete pattern is shown in Figure 5.

The alteration in the gross appearance of the liver surface in Stages IIIA and IIIB can be seen in the photographs of the median lobe (Fig. 1). The uniform degree of regeneration taking place throughout the liver in Stage III confers a uniform appearance to the organ. The surface is no longer "smooth," as it is in the normal liver. The slight pits or indentations visible on the surface of the rat liver in Stage III are produced by central veins that drain the surface cells and the thin capsule. The appearance of such livers resembles that of a regenerated liver remnant as it appears two or three weeks after a conventional (two-thirds) hepatectomy has been performed in the normal rat. In the case of the fatty liver, each acinar unit, already distended by fat-filled cells, is further increased in size by the addition of newly formed cells. Such units bulge slightly between the individual central veins draining the surface and produce a "lobular" pattern.

**Stage IV: Irregular Periportal Regeneration.**—In the fourth stage in the development of fatty cirrhosis, the liver of the rat becomes grossly nodular. The earliest evidence of this stage is the appearance of a few discrete projections on the surface of the liver (Figs. 1 and 6). During Stage III the external surface of the liver has a uniform appearance, a pattern marked off by slight, but regular, pitting of the surface. As the transition to Stage IV occurs, certain areas enlarge and project further from the surface, as nodules. This process is not limited to the surface but occurs in a haphazard fashion in all parts of the liver (Fig. 6B). This is the beginning of irregular periportal regeneration. This change, one in which certain liver acini exhibit greater hyperplasia than others, is shown in Figure 7. The newly formed parenchymal

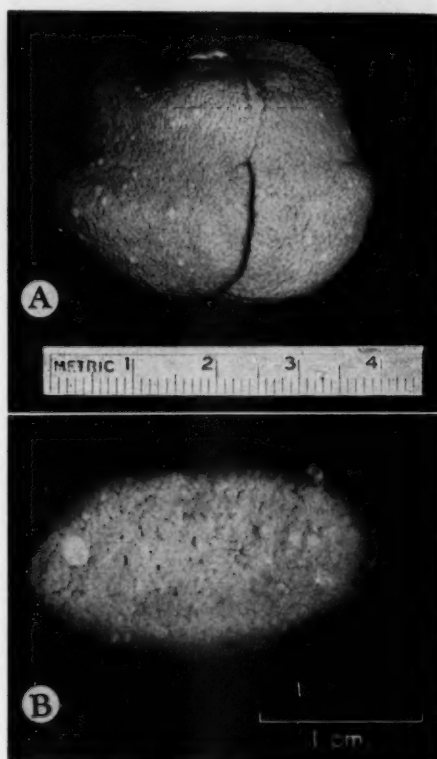


Fig. 6.—Gross appearance of regenerative nodules in Stage IVA of choline-deficiency fatty liver disease in the rat. The nodules, initially less fatty than adjacent tissue, are evident as surface projections (A). They occur in a haphazard fashion throughout the liver (B, cut surface).

cells no longer appear to be arranged in an orderly fashion and tend to assume the form of a nodule. By expansion, such nodular-cell masses distort the structure of the liver and compress nearby veins, arteries, and bile ducts.

Stage IV may be subdivided into three phases. In the earliest, or transition, phase, IVA, the regenerative nodules or hyperplastic acini are barely visible on gross inspection and appear as isolated nodules in tissue sections (Fig. 7). Stage IVB is that in which approximately one-half of the liver is made up of such nodules (Figs. 1 and 8). Eventually, the entire liver appears to consist of regenerative nodules; nearly all of the original liver structure is compressed be-

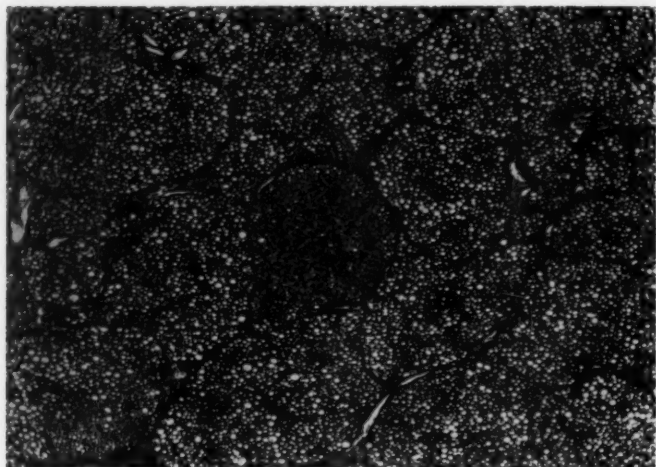


Fig. 7.—Stage IVA. In the center of the field, a compact mass of liver cells is evident. Such cells are relatively less fatty than the surrounding ones. This represents a "regenerative nodule" in an early stage of formation and is the result of irregular periportal regeneration. Hematoxylin and eosin; reduced to 85% of mag.  $\times 50$ .

Fig. 8.—Stage IVB. Four nodules (areas of irregular periportal regeneration) are evident across the center of the field. The growth of adjacent liver acini has not kept pace with these and appear to be compressed by the expansion of the nodules. Hematoxylin and eosin; reduced to 85% of mag.  $\times 50$ .

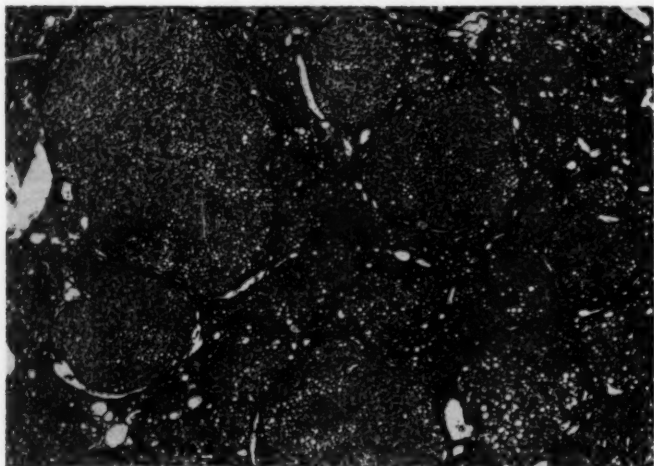
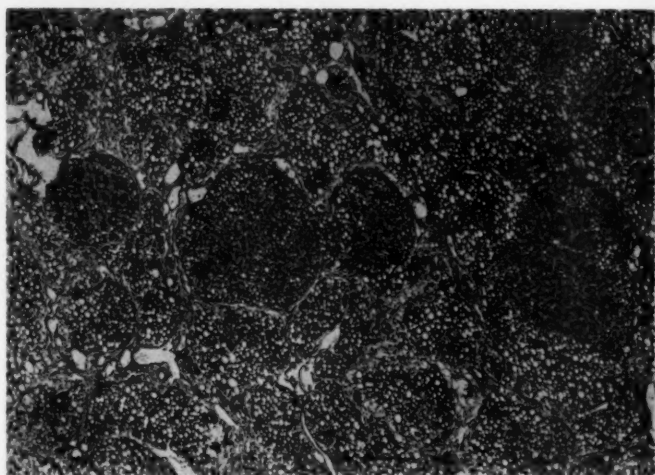


Fig. 9.—Stage IVC. The original structure of the liver has been grossly distorted and compressed by the expanding nodules. The vascular components, the bile ducts, and the residual or atrophic liver acini are compressed into the thick trabeculae. A small area of necrosis is visible in the lower pole of the largest nodule. Hematoxylin and eosin; reduced to 85% of mag.  $\times 50$ .

tween the growing nodular masses (Fig. 9). This stage has been designated as IVC. There is a decrease in the size of the liver as the last stage develops (Fig. 1).

The regenerative nodules in fatty cirrhosis represent an irregular form of new-cell growth that develops about the terminal branches of the portal vein. In the young growing rat fed a diet severely deficient in choline, the nodules that develop in Stage IV usually represent individual acini that have undergone hyperplasia, i. e., monoacinar nodules.<sup>9</sup> The small portal vein and its accompanying structures do not necessarily occupy the center of the nodule but may be located eccentrically. In advanced fatty cirrhosis some regenerative nodules are comparatively large, i. e., 2 or 3 mm. in diameter. In transverse sections of such structures several vessels are often seen within the nodule. One interpretation is that the vessel was originally a single branch arising from a conducting portal vein and has subsequently divided. Rappaport and Hiraki<sup>9</sup> offer an alternative explanation; viz., several acini may have been incorporated in the substance of a single nodule because the original deposition of fat was not entirely uniform in each and every acinus. Conducting branches of the portal vein are incorporated into the substance of the nodule only where the expanding mass of cells has grown around and has partially encircled the vein. Afferent veins are always located at the periphery of nodules and lie within the compressed trabeculae. The larger branches of the hepatic veins are compressed by the expanding regenerative nodules, often to a greater degree than the larger portal veins. This has been observed both in casts of the venous systems in corrosion preparations and in injected specimens of rat livers that have been cleared in cedar oil.<sup>10</sup> The general effect is quite similar to that described in cirrhosis in man.<sup>11,12</sup>

The cells of the regenerative nodule in experimental fatty cirrhosis in the rat are usually irregularly arranged and appear in the form of two-cell-thick plates. At times, however, the original single-cell-plate form

is maintained. Hartroft<sup>13</sup> has emphasized that the cells of the nodules are less fatty than their predecessors, the original liver cells. In general this is true (Figs. 7, 8, and 9). However, fat does eventually accumulate in the cells of regenerative nodules, and fatty cysts may form within the confines of these structures. The collapse of fatty cysts may contribute to the subsequent formation of incomplete septa within the confines of a large nodule. Several focal areas of regeneration developing independently in a large nodule may compress the disintegrating cysts between them and partially subdivide the original nodule, thereby increasing the complexity of the histologic appearance.

At times an apparently rapid necrosis of a portion of or of an entire regenerative nodule occurs in Stage IV. This is an additional factor that adds complexity to the microscopic appearance of the late stages of experimental fatty cirrhosis, since it results in collapse and approximation of the structures that formerly bounded the original nodule. The large regenerative nodule in Figure 9 contains a small area of early necrosis in its lower pole. The cause of such focal areas of necrosis is obscure. In our material, areas of cellular necrosis have been seen *only* in regenerative nodules in the choline-deficient rat. Submassive necrosis of the liver is not a feature of the earlier stages.

*Related Observations.*—Animals fed protective, or partially protective, amounts of choline were killed at intervals of one to six months. The addition of choline to the C-8 diet at a level of 0.5% (38 males) prevented the accumulation of stainable fat in the liver. Such animals grew at a rate commensurate with the restricted caloric intake (approximately 45 Cal. per day furnished by 8 gm. of diet) and remained healthy. To 17 male rats a 0.2% supplement was fed for 3 to 26 weeks; 6 showed centrolobular fat (Stage I); in 11 no fat was demonstrated. Twenty-two male rats received a 0.4% supplement for 5 to 30 weeks. Centrolobular accumulations of fat

were present in 20; none progressed beyond Stage I. Rats of the same age and strain were employed in both experiments, but the results are not strictly comparable, since the experiments were not conducted simultaneously.

The results of adding 0.05% choline to the test diet were observed in 17 rats that were killed at intervals. The results were as follows: Stage I—six rats (5, 7, and 26 weeks); Stage IIIA—three rats (26 weeks); Stage IIIB—three rats (26 weeks); Stage IVA—three rats (26 weeks). In two rats (killed at 15 and 26 weeks, respectively) the distribution of fat was irregular, and the specimens could not be readily classified. The addition of choline at the level of 0.05% retarded the rate of development of cirrhosis.

The development of cirrhosis in young rats fed Diet C-8 was found to be relatively uniform and predictable in the foregoing experiments. When the diet was fed to older rats, which grew more slowly, the hepatic changes observed were less constant, though in general the evolution of fatty liver to cirrhosis followed a similar sequence. When the original deposition of fat was irregular and less severe, the development of "cirrhosis" appeared to be irregular and inconsistent in the older rats.

### Comment

The question may be raised: What is the purpose of the suggested classification of fatty cirrhosis in the rat? Is this merely a substitution of numbers for such terms as hypertrophic and atrophic, early or late cirrhosis? One answer would be the lack of agreement in the definition of descriptive terms that are frequently employed. A difficulty inherent in the terms "early" and "late" is that the element of time is implied. In the case of the present experimental model, "time" implies the duration of action of the inciting cause, i. e., the nutritional deficiency enforced upon the animals. The architectural changes that develop in the liver at any given "time" in the course of the

experiment is a function of many variables: age at the time the diet is started, sex, genetic constitution, nature of the diet, etc. The terms hypertrophic and atrophic are more satisfactory, but there is no general agreement as to what criteria, other than size of the liver, constitute the distinguishing features. No system of "grading" cirrhosis can ever be entirely satisfactory. There is very poor correlation between the function of a liver and the alteration of its architecture, i. e., various "degrees" of cirrhosis. There is very little fundamental difference between livers graded by the present system as "late" Stage IIIB or Stage IVA. Yet if one wishes to discuss lesions in the liver that can be produced by experimental diets, some system of classification is desirable. The concept of four stages in the evolution of fatty cirrhosis in the rat has been useful in the laboratory to record the findings encountered in certain experiments.

The concept of liver acini introduced by Rappaport<sup>14</sup> to describe the areas about the terminal portal vein branches as functional units is advantageous. The demarcation of such areas in rat liver can be seen in fatty cirrhosis.<sup>6,9</sup> The functional capacity of these areas in terms of regeneration were recognized by Lillie and Ashburn and their associates in their early studies of the fatty cirrhosis in the rat, though the term acinus was not employed.<sup>7,8</sup>

The emphasis on regeneration in the present classification diverts attention from "fibrosis" in the development of fatty cirrhosis in the rat. The membranous trabeculae are considered to be relatively unimportant, though as visible fibrotic strands they furnish convenient histologic evidences by which one can recognize the presence of the pathologic process. In the rat the membranous trabeculae are very thin in Stages IIIB and IVA, a feature that readily becomes apparent when one examines sections of "arrested" cirrhosis in specimens secured from animals that have been returned to a normal diet. The delicate nature of the septa is a reflection of the fact that the

original supporting structure of the rat liver, like the capsule, is very thin. Vascular channels that traverse membranous trabeculae are admittedly important, particularly when they constitute intrahepatic shunts. However, such channels are not the result of fibrosis *per se*. Presumably they arise from original sinusoids; the position they occupy is determined by the original location of cellular disintegration, and their course through the septa is governed in part by where these structures are pushed by the forces of regeneration. Vascular injections provide little evidence that extensive vascular channels exist as shunts in the membranous septa in Stage IIIB or Stage IVA in the rat.<sup>10</sup> The absence of such a feature may explain why the liver disorder seems relatively "benign" at this stage. The presence or absence of intrahepatic shunts in experimental cirrhosis has an important bearing on the question of progression or self-perpetuation of the cirrhosis process after the initial inciting cause has been eliminated. Most forms of experimental cirrhosis fail to demonstrate self-perpetuation once the injurious factor or agent has been withdrawn.

A study of the development of fatty cirrhosis in the rat offers an opportunity to study the origin of the regenerative nodule. Stage IVB or Stage IVC represents a relatively simple form of cirrhosis when virtually every regenerative nodule is basically a monoacinar nodule. Although on a single microscopic section the nodules appear irregular and seemingly dissimilar, nevertheless they are fundamentally alike. This is apparent from a study of their origin and growth. It is an expression of the fact that the original liver-cell injury in severe choline deficiency is almost always uniform in respect to its geographic location in each and every acinar unit. The full development of experimental fatty cirrhosis is attained when regenerative nodules completely dominate the gross and microscopic picture. By their expansion the nodules compress the remainder of the original liver tissue into membranous septa of varying thickness and composition.

The development and the subsequent growth of the regenerative nodule are believed to be the most important feature in this form of experimental cirrhosis. The significance of nodular regeneration in the pathology of cirrhosis of all types has been emphasized by Baggenstoss: "Nodular regeneration holds a unique position in the pathogenesis of cirrhosis of the liver, and it is, in fact, the key to adequate understanding of this disease."<sup>15</sup> In experimental fatty cirrhosis there is no satisfactory answer to the question: Why does one acinus become hyperplastic, seemingly at the expense of the immediate adjacent areas? Admittedly, one may overemphasize the significance of the growing mass of cells and neglect the fact that the significant pathology may actually reside in the areas that do not maintain their continued growth. It might be more accurate to refer to Stage IV as irregular periportal atrophy. Viewed in that aspect, the regenerative nodule in experimental fatty cirrhosis could be regarded as a passive phenomenon, i. e., a mass of tissue permitted to grow and expand because the adjacent areas are undergoing atrophy. In the study of our material we have not observed occurrence of massive or submassive necrosis and collapse of the acini adjacent to hyperplastic areas that appear to be regenerative nodules under development. The atrophy of such acini, i. e., those that are not destined to become regenerative nodules, appears to be a slow process. The phenomenon is a difficult one to analyze. The explanation may reside in the nature of the blood supply to the individual areas. So far, a study of injected blood vessels in specimens exhibiting Stage IV patterns has not furnished satisfactory answers.

As previously indicated, spontaneous necrosis of a portion of or an entire regenerative nodule has been observed in Stages IVB and IVC, though the frequency of occurrence is not known. It is a phenomenon that must be taken into account in analyzing the ultimate architectural changes in any type of cirrhosis, as Popper and Baggenstoss have emphasized in studies on

human cirrhosis.<sup>16,17</sup> Further observations are required to determine whether necrosis of regenerative nodules continues to occur in rats after fatty cirrhosis has been "arrested" at Stage IVB or Stage IVC in animals returned to an adequate diet. This, too, has an important bearing on the question of progression or self-perpetuation of cirrhosis in the rat. The existence of intrahepatic vascular shunts and the occurrence of spontaneous necrosis of regenerative nodules may be associated.

### Summary

1. Four stages in the evolution of fatty liver disease to cirrhosis in the choline-deficient rat can be recognized. The histologic patterns can be seen to best advantage in young, rapidly growing rats maintained on diets that induce heavy accumulations of fat evenly distributed throughout the entire liver. Histologic features permit grading of such specimens in four stages: Stage I (centrolobular fat): Intracytoplasmic fat accumulation involves approximately one-half the parenchymal cells. Stage II (entire lobule fatty): Virtually every parenchymal cell is involved. Stage III (uniform periportal regeneration): Newly formed cells are relatively equally distributed in each acinar unit. Stage IV (irregular periportal regeneration): Regenerative nodules, erratically distributed throughout the liver, form by hyperplasia of individual acini.

2. In the choline-deficient rat, a hypertrophic fatty cirrhosis exists in Stage III, and an atrophic fatty cirrhosis exists in Stage IV. The pressures exerted by individual areas of regeneration of parenchyma are believed responsible for the pattern of the fibrous trabeculae evident in microscopic sections.

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# Fatty Cirrhosis in the Rat

## II. Measurements of Liver-Cell Regeneration

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The present study was undertaken to measure the magnitude of liver-cell regeneration during the development of fatty cirrhosis in rats maintained on a choline-deficient diet. The degree of regeneration was estimated by two methods, viz., (a) mitotic counts, using the Feulgen nuclear stain, and (b) measurements of the rate of  $P^{32}$  incorporation into the deoxyribonucleic acid (DNA) of the liver cell in a three-hour period.

### Material and Methods

Male rats of a uniform stock (35 days old; body weight 80 to 105 gm.) were placed on the test or control diet on the day of arrival at the laboratory. The animals were kept in individual cages in a continuously illuminated room. Two individual groups of 60 rats (48 test and 12 control animals) were started on the diets 10 weeks apart. The test rats were maintained on a hypolipotropic diet, designated C-8; the composition is described in the preceding paper.<sup>1</sup> This diet is a high-fat, low-protein diet that regularly produces a severe degree of fatty liver in young, rapidly growing rats. The control animals received the same diet supplemented with 0.5% choline chloride (Diet C-8-0.5); choline chloride replaced 0.5% of the sucrose content of the C-8 diet. Previous observations, as well as the results of the present studies, indicated that this degree of supplementation was adequate to prevent fatty-liver disease in rats under the conditions of our experiments. Each control and test animal received an allotment of 8 gm. of the diet per day. Feeding allotments were distributed three times a week. The animals were not

pair-fed; they were allowed access to food until the time of  $P^{32}$  administration. Food consumption was not measured until the day prior to the  $P^{32}$  injection. Then the animals to be killed were weighed every 12 hours, and their food consumption per 12-hour period was measured until they were injected, either at 7 a. m. or at 7 p. m.

The rats to be killed each week were arranged in an alternate sequence as regards the duration of feeding; i. e., the first group was killed after 1 week on the diet, the second group after 11 weeks, the third group after 3 weeks, the fourth group after 13 weeks, etc. Groups of eight test rats and two controls were killed after being kept on the diet for 1 to 15 weeks; four test rats and two controls, after 16 weeks, and three test rats and one control, after 22 weeks. A total of 71 experimental rats and 19 controls were studied in these experiments. Four animals on choline-deficient diet and one control animal were injected intraperitoneally with  $P^{32}$  at 7 a. m., then killed at 10 a. m. A similar group was similarly injected at 7 p. m., then killed at 10 p. m. the same day. The animals were studied at two intervals 12 hours apart in order to detect any diurnal fluctuation in the regenerative activity that might occur.

Each test animal received 1  $\mu$ c. of  $P^{32}$  per 100 gm. of body weight, and each control animal, 2  $\mu$ c. of  $P^{32}$  per 100 gm. of body weight. At the time of killing, the animals were anesthetized with ether and exsanguinated by cutting the abdominal aorta. The liver was immediately removed, weighed, and then prepared for histological and biochemical studies. Small slices of the liver from the left lateral lobe were fixed in alcoholic Bouin's fluid and in neutral formalin solutions. The Bouin-fixed material was sectioned at 5  $\mu$  and stained by the Feulgen nuclear stain, the azocarmine triple stain and the hematoxylin-eosin stain, as modified by Harkness.<sup>2</sup> Formalin-fixed material was used for frozen sections; these were cut at 12  $\mu$  and stained with oil red O or Sudan III.

### Estimation of Regeneration

**A. Mitotic Counts.**—All counts were performed according to a procedure previously employed in the estimation of regenerative activity following partial hepatectomy in normal rats.<sup>3</sup> Colchicine was not administered. Slides were prepared with every

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third section from the paraffin ribbon mounted in order to reduce the error of counting the same cell twice when the sections were examined. The number of mitoses seen in 100 high-power fields ( $\times 43$  objective dry and  $10\times$  ocular) was recorded. These fields were selected at random from each specimen. The errors inherent in estimating the magnitude of liver-cell regeneration by counting the number of cells in mitosis are appreciable. Fairly accurate results can be obtained in rapidly regenerating liver when the cells are quite uniform in size and distribution, as is the case in a normal rat subjected to the customary partial hepatectomy. However, when the liver tissue is distorted by varying degrees of fat accumulation and by the structural disorganization that characterizes cirrhosis, the problem becomes much more complicated, and the accuracy of the estimations is further reduced. In a fatty liver or in a fatty cirrhotic liver, the total number of parenchymal cells visible in a single high-power field is less than in sections from a normal animal. It was necessary to establish a correction factor in order that the number of mitoses counted in 100 fields in the various specimens could be expressed on a uniform basis. Liver sections from the control animals that received diet C-8-0.5 (choline-supplemented) were taken as the standard of reference. The parenchymal cells in such specimens are virtually free of visible fat droplets, though the cytoplasm is vacuolated. The total number of parenchymal liver cells in 10 high-power fields from sections of 10 different livers of the control group were counted. In these specimens the average number of cells per high-power field was 152. In a similar fashion, figures were obtained from representative slides for Stage II, IIIA, IIIB, IVA, and IVB of fatty cirrhosis encountered in this study. The liver in Stage II or Stage III presents a fairly uniform appearance; random selection of any area (excluding large blood vessels) probably gives a fair representation as to number of cells in a given field. In Stage IV, the presence of regenerative nodules complicates the picture because the cells of the nodules are often less fatty and may be more compact, especially when they are composed of two-cell-thick plates. The areas chosen for total cell counts for Stage-IV specimens were therefore selected in order to give a known distribution of areas that were entirely limited to a nodule ("nodular"), covered a portion of a nodule ("mixed"), or contained no clearly recognizable nodule ("non-nodular"). In order to conform to the random distribution that had been recorded when the original mitotic counts per 100 high-power fields were performed on the Stage-IV slides, the following distribution was employed for the total cell counts: for Stage IVA: nodular 14%, mixed 10%, non-nodular 76%; for Stage IVB:

TABLE 1.—Factors Employed to Express the Number of Mitoses Counted in One Hundred High-Power Fields to "Mitoses per 100,000 Cells"

Specimen	Average No. of Parenchymal Cells per High-Power Field	Factor
Control	152	$\times 6.6$
Stage II *	135	$\times 7.3$
Stage IIIA †	126	$\times 7.9$
Stage IIIB †	113	$\times 8.8$
Stage IVA †	107	$\times 9.4$
Stage IVB †	120	$\times 8.3$

\* On basis of ten random fields from each of eight slides.

† On basis of ten random fields from each of ten slides.

‡ Areas selected (see text).

nodular 41%, mixed 29%, non-nodular 30%. By these means, an attempt was made to correct the mitotic counts for the variations in the total number of parenchymal cells scanned in the different stages. In order that the number of mitoses observed in 100 high-power fields of this heterogeneous material might be expressed on a uniform basis, the figure "per 100,000 cells" was used. The factors necessary for this conversion were derived as shown in Table 1. The inherent limitations in the accuracy of this estimation of mitotic counts is recognized. The data obtained represent a semiquantitative measurement of the regenerative activity in the liver.

**B. Biochemical Procedures.**—The total liver was weighed immediately on removal. After small sections were taken for the histological examinations, the remaining liver tissue was reweighed. The tissue was placed in a small tissue press and forced through holes about 1 mm. in diameter; the stroma and connective tissue was left behind. The pressed tissue was homogenized in a glass homogenizer with cold 2% citric acid and the homogenate made to 25 cc. A 1 or 2 cc. aliquot of this homogenate was added to an equal volume of cold 10% trichloroacetic acid (TCA) for subsequent evaluation of total ribo- and deoxyribonucleic acid (RNA and DNA), as well as the specific activity of the acid-soluble phosphorus (ASP).

From the remainder of the homogenate, DNA was isolated and purified so that its specific activity could be measured. This was accomplished by the following steps: The nuclear fraction was centrifuged down from the homogenate, and the process of suspension in cold 2% citric acid and sedimentation was repeated two more times. The sediment was next heated in a boiling water bath with a small volume of 6% NaCl buffered at pH 10 with  $\text{NaHCO}_3$  and  $\text{Na}_2\text{CO}_3$  in order to coagulate the protein, depolymerize the RNA, and solubilize the DNA. The soluble DNA was collected with the

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TABLE 2.—Mitotic Indices in Control (Choline-Supplemented) and in Choline-Deficient Rats with Various Stages of Fatty Liver Disease

No. of Rats	Diet	Weeks on Diet	Status of Liver	Mitotic Index *	
				Range	Average
19	C-8-0.5	1-22	Normal	0-13	6
8	C-8	1	Stage II	30-343	102 †
14	C-8	3-5	Stage IIIA	33-181	108
23	C-8	5-9 (18 rats) 11-15 (5 rats)	Stage IIIB	33-229	102
16	C-8	11-15	Stage IVA	75-244	152
10	C-8	15-22	Stage IVB	83-183	120

\* Mitoses per 100,000 cells.

† See text and Figure 1.

supernatant fluid after centrifugation and was then precipitated by the addition of 2 vol. of 95% ethanol. The precipitated, but not yet pure, DNA was reextracted with 10% NaCl in a boiling water bath and reprecipitated with ethanol. This precipitate was then heated at 85 C for 30 minutes with 0.1N NaOH, to hydrolyze any remaining RNA, cooled in an ice bath, and made to about pH 1 with HCl and to 5% with TCA. After standing one hour in the cold, the DNA was centrifuged down, washed with cold 5% TCA, and finally hydrolyzed with 5% TCA at 85 C for 30 minutes. This hydrolyzed DNA was filtered into Pyrex test tubes, wet-ashed with H<sub>2</sub>SO<sub>4</sub> and HNO<sub>3</sub>, and finally made to a convenient volume (generally 5 cc.) for subsequent estimation of P<sup>32</sup> and total phosphorus.

The aliquot of the homogenate in TCA was left cold one hour, centrifuged, and the supernatant fluid filtered into a Pyrex tube for wet ashing and subsequent evaluation of the specific activity of acid-soluble phosphorus. The TCA precipitate was washed with cold 5% TCA, extracted first with 95% ethanol, then three times with boiling ethanol-ether (3:1), and finally again with 95% ethanol. This lipid-free residue was then extracted with 5% TCA at 85 C for 30 minutes and again for 15 minutes. The hot TCA extracts were pooled,

made to a convenient volume (usually 10 cc.), and used directly for the colorimetric determination of DNA by the diphenylamine reaction<sup>4</sup> and of RNA by the orcinol-HCl reaction.<sup>5</sup>

Radioactive phosphorus (P<sup>32</sup>) in the DNA and ASP fractions was measured with a dipping Geiger-Müller tube coupled to a scaling unit, and total phosphorus of these same solutions was evaluated by the method of Fiske and Subbarow.<sup>6</sup> Relative specific activity as reported in this paper was calculated as follows:

$$\frac{\text{Counts/min./}\mu\text{g. DNA-P}}{\text{Counts/min./}\mu\text{g. ASP}} \times 100$$

## Results

The data obtained in this study are presented in Tables 2 and 3 and are depicted in graphic form in Figures 1 and 2. The livers of the control animals appeared normal; histologic examination revealed only an occasional small fat droplet in the parenchymal cells in a few of the frozen sections. In the hematoxylin-eosin sections, the cells did exhibit the vacuolated or hypocytoplasmic appearance characteristic of the protein-deficiency state. The control rats had

TABLE 3.—Biochemical Data in Control (Choline-Supplemented) and in Choline-Deficient Rats with Various Degrees of Fatty Liver Disease

Stage	No. of Rats	Time, Wk.	Liver Weight, % of Body Wt.	Nucleic Acid, Mg. Per 100 Gm. Body Weight		Ratio of DNA:RNA	Relative Specific Activity of DNA	Mitotic Index
				RNA	DNA			
Control (Group 1)	9	1-9	4.5	35.8±1.4	10.7±0.7	0.30	0.18±0.3	6
Control (Group 2)	8	11-22	3.9	30.4±1.2	7.3±0.2	0.24	0.24±0.3	6
Stage II	8	1	6.2	32.6±1.2	9.4±0.5	0.29	1.5±0.45	102
Stage III	14	3-5	7.5	37.2±1.6	13.6±0.7	0.37	1.86±0.30	105
	23	5-9 11-15	6.6	32.7±0.9	16.9±0.7	0.52	1.45±0.11	102
Stage IV	16	11-15	5.9	31.7±1.2	17.7±0.9	0.56	0.78±0.09	182
	10	15-22	5.0	31.0±1.3	17.3±1.3	0.56	0.78±0.09	120

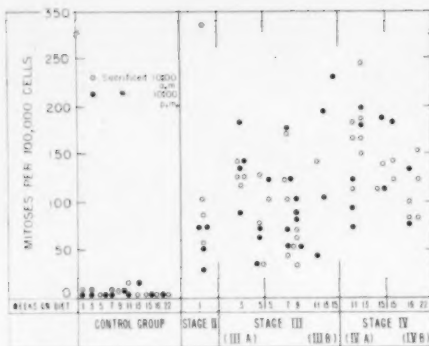


Figure 1

Fig. 1.—Mitotic indices in control (choline-supplemented) and choline-deficient rats killed at intervals of 1 to 22 weeks.

Fig. 2.—Average values for mitotic index, RNA/DNA ratio, and relative specific activity of

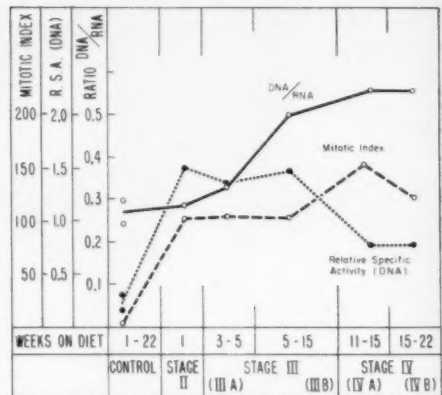


Figure 2

DNA in control (choline-supplemented) and choline-deficient rats killed at intervals of 1 to 22 weeks.

very low mitotic indices; a value of 6 or 13 per 100,000 cells indicates that only one or two dividing nuclei could be found in 100 fields. For the presentation of biochemical data in Table 3, the control rats have been divided into two groups, those killed at 1, 3, 5, 7, and 9 weeks (Group 1) and those killed at 11 to 22 weeks (Group 2). The increase in body weight in the animals in Group 2 (average, 84 gm. as starting weight, 136 to 236 gm. when killed, 11 to 22 weeks later) must be taken into account in evaluating the available data. The figure for the liver weight, when expressed as the percentage of body weight, is less in the older and heavier animals of control Group 2 (3.9%, as compared with 4.5% for Group 1).

The livers from the 71 test animals were graded as to the state of fatty liver on the basis of the histologic appearance, employing the criteria described in the preceding paper.<sup>1</sup> They were classified as follows: Stage II, 8 rats; Stage III, 37 rats, and Stage IV, 26 rats, as indicated in Table 2. The relationship of the stage of liver disease to the time of killing is shown in Figure 1. The rats employed in this study were young (35 days of age); they reached Stage II (with virtually every liver cell containing fat droplets) rapidly. All eight rats in the

first week's experiment were found to be in the second stage. The animals killed after three, five, seven, and nine weeks on the diet were classified as Stage III (i. e., histologic evidence of uniform periportal regeneration). The division of Stage III into an early phase (IIIA,) in which trabeculae are inconspicuous, and a late phase (IIIB), in which the trabeculae become increasingly prominent, is chiefly of interest from a morphogenetic standpoint. The presence or absence of "fibrosis" in Stage III of fatty cirrhosis in the rat is believed to be relatively unimportant from a functional standpoint. The majority of the animals killed after 11 weeks on the choline-deficient diet exhibited the characteristic changes of Stage IV, viz., morphological evidence of irregular periportal regeneration (regenerative nodules). Five rats among those killed at 11, 13, and 15 weeks did not exhibit evidence of regenerative nodules and were therefore classified as still in Stage IIIB (Fig. 1).

The average mitotic index of the eight rats in Stage II was 102 (Table 1). It is evident from Figure 1 that the value of 102 may not be an accurate reflection of the usual intensity of the regenerative activity at this early period. The value for one animal was unaccountably high, i. e., 343.

The average value for the other seven rats was 69. The average indices were 105 and 102 for the 37 rats in Stage III (32 rats in the 3-, 5-, 7-, and 9-week groups and 5 among the 11-, 13-, and 15-week animals). Thus, throughout Stage III, when active regeneration is believed to proceed uniformly about each periportal area, the mitotic activity appeared to be relatively constant. There was wide fluctuation in the number of mitoses noted from one specimen to another in all groups. This is evident in Figure 1. The average mitotic index for the rats killed after 11 weeks was higher than in the earlier groups. The average value for the 16 rats classified in Stage IVA was 152; a somewhat lower figure, 120, was found for the 10 rats in Stage IVB.

In the course of performing the mitotic counts on the 100 high-power fields of the slides graded Stage IV, each randomly selected area was recorded as "nodular," "non-nodular," or "mixed." In the specimens graded IVA, the percentage distribution was 14, 76, and 10, respectively. In the Stage IVB specimens, the corresponding distribution of the areas selected at random was as follows: "nodular," 41%; "non-nodular," 30%, and "mixed," 29%. Mitotic figures were found somewhat oftener in the nodular areas, but they were not limited to them. In Stage IVA, 24% of the mitoses observed in the random counts were in the high-power fields that contained only cells of a regenerative nodule. The non-nodular areas accounted for 63.3%, and the mixed areas for 12.7%, of the mitoses observed. In the 10 specimens graded IVB, 59% of the mitoses were found in areas that consisted exclusively of cells from a regenerative nodule, 21% were in the mixed areas, and 20% in the non-nodular areas. There are usually more liver cells in a given microscopic area of a nodule than from a similar area of a non-nodular portion. The cells in a nodule are usually less fatty and more compact. As noted earlier, an attempt was made to correct for this variable in the factor employed to express the number of mitoses on a uniform basis.

The biochemical data are presented in Table 3. As mentioned above, the data from the control rats have been presented separately for the animals in the first half (9 weeks) and for those in the second half (11-22 weeks).<sup>\*</sup> There was a prompt increase in the weight and size of the liver within one week of the start of the choline-deficient diet. The fat content of the livers of such animals may be as high as 20% to 40%. Although the body weights of the test animals killed early were similar to those of the controls, the livers in Stage II and Stage III were 6.2% to 7.5% of the total body weight, in contrast to 4.5% for the control group. This is a reflection of the increased fat content plus some addition by new-cell formation. In Stage IV, the liver takes the form of an atrophic nodular cirrhosis, and the liver weight again declines. The liver weight was 5% of the body weight in Stage IVB. Such livers contain less fat. The amount of RNA, expressed as milligrams of nucleic acid per 100 gm. of body weight, remained quite constant (30.4 to 35.8 in the control group, 31.0 to 37.2 in the experimental animals). The progressive rise in the total amount of DNA (Table 2) is believed to be significant. In the process of fatty-cyst formation many of the liver-cell nuclei appear to remain intact when the cell membranes rupture. When the fatty cysts collapse, the nuclei and the cyst-wall remnant of some become incorporated in the trabeculae. At times, the cyst remnants resemble bile ductules.<sup>7</sup> The increase in total DNA content of the liver may reflect the survival of some original nuclei plus the formation of additional nuclei of the newly regenerated cells. The ratio of DNA to RNA (Table 2) rose until Stage IV was attained; then the ratio appeared to remain constant. The contribution of an increase in the number of nonparenchymal cell nuclei (endothelial or littoral, bile duct, supporting framework) to the total DNA content is

<sup>\*</sup> Biochemical data were lost (laboratory mishap) on two control rats (1st and 13th weeks); hence data available on 17 of 19 rats are presented in Table 3.

difficult to assess. These elements, estimated by Harkness to represent 30% to 40% of the nuclei in the normal liver,<sup>8</sup> may increase somewhat in fatty cirrhosis. However, it seems unlikely that this would account for the increases in DNA that were noted. Cellular infiltration by neutrophils or lymphocytes did not appear to be a prominent feature in fatty liver disease in the rats studied in these experiments.

The average value for the relative specific activity of the DNA

$$\left( \frac{\text{Counts/min/}\mu\text{g. DNA}-P}{\text{Counts/min/}\mu\text{g. ASP}} \times 100 \right)$$

was 0.18 in the first group of control animals and 0.24 in the second group. As can be seen in Figure 2, a marked increase in the relative specific activity was noted in the rats in Stage II (1.50). High values were also obtained in the rats in Stage III (1.36 and 1.45). These values represent a sevenfold increase over those obtained in the control rats. This increase in activity was observed despite the fact that the total metabolic pool of DNA in these fatty livers was increasing (Table 3). In Stage IV, no further increase in total DNA was observed, but the relative specific activity of the DNA declined sharply (1.45 to 0.78).

Inspection of Figure 1 will reveal that no significant difference was noted in the mitotic activity in the rats killed at 10 a. m. and those killed at 10 p. m. The biochemical data failed to show a significant evidence of periodicity when it was similarly analyzed. The studies of Jaffe<sup>9</sup> on regenerative activity in rats subjected to partial hepatectomy revealed a striking diurnal fluctuation in mitotic counts; high values were noted in the morning and low values at night. Barnum et al.<sup>10</sup> also observed diurnal fluctuations in mitotic activity and in the specific activity of DNA in the growing livers of immature mice. It is not surprising that mean values obtained in the present study failed to demonstrate evidences of diurnal fluctuation. The livers were extensively involved in a pathological process; the rate of progression was not entirely uniform. Measurements of the food consumed by the

animals during the 36 hours that preceded the P<sup>32</sup> injection failed to demonstrate any consistent feeding pattern. Since the animal room was continuously illuminated, the factor of alternate light and dark did not exert an influence that would tend to synchronize periodic fluctuations that might have existed in individual animals.

### Comment

The ability of the liver to restore itself whenever its total function is threatened by death of cells or partial removal is well known. The statement of Ravdin and Vars,<sup>11</sup> "We know of no other organ in the body that possesses such an irresistible urge to regenerate after injury even under unfavorable circumstances" is underscored by observations they have made in their own laboratory. Partial hepatectomy, a procedure in which the rat is suddenly deprived of two-thirds of the liver, has been utilized by many investigators to study this regenerative response.<sup>8</sup> The recent report of Ingle and Baker<sup>12</sup> emphasizes the remarkable ability of a normal rat to regenerate liver tissue despite repeated partial resections. In studies of this regenerative capacity, investigators have created a number of "unfavorable circumstances," among which may be mentioned common-bile-duct ligation,<sup>13,14</sup> hepatic-artery ligation,<sup>15,16</sup> and portal-vein ligation.<sup>17</sup> Despite these experimentally induced handicaps, the rat is usually able to respond to partial hepatectomy and regenerate liver tissue. The ability to respond to partial hepatectomy has been demonstrated in animals fed protein-deficient or choline-deficient diets.<sup>18</sup> It is not surprising, therefore, that the methods employed to measure new liver-cell formation in the current study revealed increased values. The regeneration that follows the classical hepatectomy in the rat is sudden and very striking for a short period. Then it subsides. In the course of the development of cirrhosis in the rat, regenerative activity is less dramatic at any one moment, but it is one of considerable magnitude, since it represents a continuing

process extending over a period of many weeks.

The biochemical data obtained in the present study of 71 rats maintained on a choline-deficient diet for periods of 1 to 22 weeks reflect the ability of the liver to maintain certain aspects of its chemical composition in the presence of unfavorable circumstances. In such livers, the parenchymal cells become so engorged with fat that they rupture, and the normal architecture becomes distorted by the formation of trabeculae and membranous septa. Despite these handicaps, the experimental rats maintained an over-all content of ribonucleic acid very similar to that of the control animals. The average amount of nucleic acid in the form of RNA, when expressed in relation to body weight of the rat (Table 2), remained relatively constant, though the structure of the liver was markedly deranged. The amount of deoxyribonucleic acid steadily increased, until the fourth stage of fatty cirrhosis was reached. The formation of fatty cysts, structures composed of ruptured cell walls but with intact nuclei, could account for this observation. These altered cells presumably contain reduced amounts of cytoplasm. They can survive, as Hartroft and Sellers<sup>10</sup> demonstrated. The intact nuclei of such liver cells become incorporated into the trabeculae and may be considered to be in a resting state. It seems unlikely that the DNA content of the liver would increase during the development of fatty cirrhosis if necrosis and death of an appreciable number of individual liver cells had occurred. Hartroft has stated that acute necrosis of parenchymal cells is hardly ever seen in this disorder.<sup>20</sup> Other observers have placed more emphasis on the importance of cellular and focal degeneration (necrosis), as György has indicated in a recent review.<sup>21</sup> The histologic evidence of acute liver-cell necrosis is minimal in the examples of fatty liver disease we have studied. Once Stage IV of fatty cirrhosis is reached, submassive necrosis is occasionally observed, but only in the *regenerative nodules*. This form of necrosis is a late phenomenon and

is not believed to be a factor in the pathogenesis of the early stages of the disorder.<sup>1</sup>

The rate of incorporation of radioactive phosphorus into the DNA was distinctly increased in the 71 experimental animals. The relative specific activity of the DNA, elevated within one week of the time the liver became engorged with fat, reflected the prompt regenerative activity that ensued as soon as the liver was threatened by loss of available functioning tissue. The relative specific activity remained high throughout Stage III, i. e., until the 13th to the 15th week. As regeneration in the liver became irregular, Stage IV, the rate of formation of DNA appeared to diminish. The total amount of DNA in Stage IV was approximately the same as that in Stage IIIB, but the relative specific activity decreased from 1.4 to 0.78. The value of 0.78 was distinctly higher than that of the control animals (0.24 in the controls killed after 11 to 22 weeks). Unfortunately, very few data are available in the very late stages (Stage IVC) of experimental fatty cirrhosis. A single animal, a male maintained on the C-8 diet for 27 weeks, was investigated. In this rat the relative specific activity of the DNA was 0.5; this observation was not included in the data presented. The specimen was graded IVC; i. e., virtually the entire liver consisted of regenerative nodules. The mitotic index, approximately 50 in this animal, was also lower than that of the rats in the earlier phases of Stage IV.

The inaccuracies inherent in the mitotic count as a quantitative measurement of liver-cell regeneration are recognized. The alteration in the histologic structure of the liver as the process of cirrhosis development proceeds is great. Although the tissue specimens employed for the counting procedure were uniform (a longitudinal section parallel to the long axis of the left lateral lobe), the number of cells in mitosis varied greatly from one specimen to another. This was true even though the general morphology, i. e., stage of fatty liver disease, was similar. The areas surveyed, 100 high-power fields selected at random, admittedly represent only

a very small sample, and perhaps greater accuracy could have been achieved with multiple sections from various liver lobes. The general trend of the mitotic index was parallel with the biochemical data until Stage IV was reached, when a discrepancy appeared, as Figure 2 illustrates. The mitotic index increased in Stage IV, whereas the relative specific activity of the DNA indicated a decrease in regenerative activity. It is possible that some regenerating cells in Stage IV may remain in mitosis for a longer time than in the early stages. Many large cells and cells with large or bizarre nuclei are present. There is no assurance that the duration of mitosis in such cells is the same as that encountered after partial hepatectomy in the normal rat. In fact, Harkness<sup>7</sup> has indicated that the commonly accepted mean duration of mitosis of approximately one hour may require modification in view of recent appreciation of the marked diurnal variation in the rate of mitosis demonstrable in rapidly regenerating liver.

The system of grading liver specimens employed in the present study is based upon morphological criteria. The distinction between Stage III (uniform periportal regeneration) and Stage IV (irregular periportal regeneration) is dependent upon the presence or absence of an anatomical feature, the regenerative nodule. The observation that biochemical differences, i. e., differences in the rate of DNA synthesis, could be demonstrated in the two stages suggests that the division is not just an arbitrary one. A fundamental difference in the mechanism of liver-cell regeneration may be operative in the formation of the regenerative nodule.

### Summary

The magnitude of liver-cell regeneration was estimated at intervals during a 22-week period in 71 rats with fatty liver disease due to choline deficiency. Nineteen rats fed a choline-supplemented diet served as controls.

The mitotic index (mitoses per 100,000 parenchymal cells) rose rapidly and remained increased as fatty cirrhosis developed in the test rats.

The RNA content of the fatty livers remained relatively constant despite progressive alterations in the architectural structure of the organ. The DNA content progressively increased until the fourth stage of fatty liver disease (irregular periportal regeneration) was reached. The synthesis of DNA was measured by estimating the rate of P<sup>32</sup> incorporation in a three-hour period. The relative specific activity of the DNA increased during the early stages of fatty liver disease, but declined as Stage IV was reached.

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# Pathogenesis of Phlebosclerosis

## *I. Phlebosclerosis of the Portal Vein*

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Phlebosclerosis of the portal vein has mostly been described in isolated case reports, and, with the exception of Li's<sup>1</sup> study, no systematic study of sclerosis of the portal vein has been reported. Simmonds<sup>2</sup> found sclerosis of the portal vein in cirrhosis of the liver and in "interferences" of the circulation. He believed the sclerosis was due to a direct effect by destruction of the liver. He also thought that there was a primary sclerosis due to previous inflammation, chiefly syphilitic. Benda<sup>3</sup> also described portal vein sclerosis in hepatic cirrhosis. McMichael<sup>4</sup> was the first to suggest that portal vein sclerosis was caused by an elevation of pressure in the portal system. Li's<sup>1</sup> study is the latest (1940). He found thickening of the intima and hypertrophy of the muscle in 26 cases, all associated with a localized increased venous pressure in the vein. He believed the sclerotic changes represented an adaptive measure to the venous hypertension. Phlebosclerosis of the portal veins has frequently been regarded as the "cause" of "Banti's syndrome"<sup>5,6</sup>; but since this syndrome is now viewed as the result of portal hypertension, it has lost its nosological status and the "cause" has been converted into an effect.

The purpose of this study, a part of a survey of phlebosclerosis in general, is to determine under what circumstances phlebosclerosis of the portal vein arises and its pathogenesis.

Sections were immediately placed in 10% formalin and stained with eosin and hematoxylin and with the Van Gieson-Weigert

method. The best differentiation by all odds was obtained with the Van Gieson-Weigert stain. The diagnosis of phlebosclerosis was made when there was any collagenous thickening of the intima. In contrast to arteriosclerosis, reduplication of the elastica was only rarely observed in phlebosclerosis from whatever source. When any marked thickening was observed, the sections were stained with Sudan IV. Lipid was not found in a single instance. The comparative absence of lipid deposits in phlebosclerosis is acknowledged.<sup>7</sup> Lipid deposits in veins are occasionally seen in experimental atherosclerosis induced by high cholesterol feeding<sup>8</sup> when the hypercholesteremia attains elevations that are not approached in humans, even in diseased conditions. The only valid explanation for the scarcity of atheroma in veins is the circumstance that the intravascular pressure in the venous system is much lower than in the arteries, being close to zero in millimeters of mercury, and even in venous hypertension the pressure never even approaches the normal intrarterial pressure. The evidences that prolonged intravascular pressure, both normal and increased, is a vital factor in the production of atherosclerosis have already been submitted.<sup>9</sup> While hypertrophy of the muscle in phlebosclerosis of the portal vein has been reported by a number of observers,<sup>1-4</sup> I have not been able to satisfy myself that this exists because of the impossibility of obtaining controls. The normal thickness of the muscular coat in veins varies. Certainly the muscle in cases of sclerosis is no thicker than in normal veins. In this opinion Page and Allen<sup>10</sup> concur.

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### Incidence of Phlebosclerosis of the Portal Vein

First, a pilot study in 100 consecutive autopsies showed that phlebosclerosis was never observed unless a hypertension of the portal circulation could be predicated. In all other conditions thickening of the intima was never noted and the vessel was perfectly normal, even when the person lived to the most advanced years. On the other hand, in portal obstruction phlebosclerosis occurs at any age—in one case in a child of 7½ months, the victim of congenital atresia of the bile ducts. Actual measurements of the portal pressure in clinical disorders have been made by Whipple and his collaborators.<sup>11</sup> Hypertension of the portal circulation was observed in the cirrhotoses, congenital and acquired obstruction of the portal or splenic veins, schistosomiasis, and other conditions that are classified under the general rubric of "Banti's syndrome." Nevertheless, on purely physiological grounds, any prolonged venous hypertension of the general circulation could simultaneously elevate the pressure in the portal circulation. We refer to any disorder that may lead to congestive failure; for instance, constrictive pericardium, emphysema, mitral disease, cor pulmonale, hypertensive disease, and, the commonest of all, coronary disease. In addition, hypertension of the portal circulation was deemed potential when intrahepatic obstruction was caused by extensive neoplastic involvement, both primary and secondary, or by an extensive gross capillary obstruction in the hepatic parenchyma, as in amyloidosis.

A systematic survey of the portal vein was therefore begun in 1953, which included all hepatic and cardiac disorders of whatever nature, whether death was directly traceable to the disease or not.\*

In all, 105 cases lent themselves for study. Results are shown in Table 1. In summary, the only causes of phlebosclerosis in the portal vein, therefore, are congestive failure

TABLE 1.—Causes of Portal Phlebosclerosis

	Number	Per Cent
Congestive failure.....	57	54.3
Laennec's cirrhosis.....	23	21.9
Laennec's cirrhosis with hepatoma.....	8	7.6
Biliary cirrhosis.....	3	2.9
Coarse nodular cirrhosis.....	3	2.9
Primary carcinoma of liver.....	2	1.9
Secondary carcinoma of liver.....	2	1.9
Amyloidosis of liver.....	1	0.9
Laennec's cirrhosis with congestive failure..	1	0.9
Chronic intrahepatic cholangitis.....	1	0.9
Congenital atresia of bile ducts.....	1	0.9
Amyloidosis of liver with congestive failure..	1	0.9
Hemangioendothelioma of liver.....	1	0.9
Cavernous transformation of portal vein.....	1	0.9

and intrahepatic or extrahepatic portal obstruction. Whatever the cause of the collagenous intimal thickening, it is histologically identical in the two conditions; but, for reasons which we shall submit later, it is more intense in portal obstruction than in congestive failure. There is one important difference. In portal obstruction the lesion is localized to the portal vein and its tributaries, whereas in congestive failure the phlebosclerosis is but part and parcel of a systemic general phlebosclerosis, as evidenced by the simultaneous involvement of the inferior vena cava.

The only reason that congestive failure is commoner in this series is because it is a commoner cause of death. The collagenous thickening of the intima in congestive failure is usually not very pronounced and may not involve even the entire lining of the vein. That the portal hypertension in congestive failure was fairly well maintained is shown by a number of particulars: First, there is usually a history of prolonged failure, as shown by the frequently recurrent attacks and by repeated hospital admissions; second, the venous pressure when taken was nearly always elevated or was strongly suggested by the finding of a positive hepatogastric reflux. We believe that the degree of the elevation of the venous pressure in the genesis of phlebosclerosis is not nearly as vital as its prolongation. For obvious reasons, it is not feasible to measure portal hypertension in congestive failure. One is

\*I am indebted to Dr. Kondo for helping me to obtain the material.

TABLE 2.—Causes of Congestive Failure

	Per Cent
Coronary occlusion.....	26.3
Hypertensive heart disease.....	8.8
Coronary disease and mitral valvulitis.....	5.3
Mitral disease and aortic insufficiency.....	1.8
Mitral stenosis.....	26.3
Mitral stenosis and aortic insufficiency.....	8.8
Coronary occlusion and emphysema.....	3.5
Mitral disease, aortic insufficiency, emphysema.....	1.8
Coronary insufficiency and diabetes.....	1.8
Coronary insufficiency.....	3.5
Tricuspid insufficiency.....	1.8
Malignant nephrosclerosis.....	3.5
Coronary occlusion and amyloidosis.....	1.8
Hamman-Rich disease.....	1.8
Constrictive pericardium.....	1.8
Aortic stenosis.....	1.8

justified, therefore, in questioning how we can assume that a generalized systemic venous hypertension may be transmitted into the portal circulation, since the liver, with its two sets of capillaries, is interposed between the right heart and the portal vein. On purely physiological grounds, we can infer that the portal hypertension may arise because of the resistance to the portal flow engendered by the increase in pressure in the hepatic vein, transmitted downward from the right heart. That the pressure in the hepatic veins is actually increased was shown in our demonstration, years ago, that phlebosclerosis of the hepatic vein was common in conditions characterized by prolonged venous pressures and congestive failure in hypertension of the pulmonary circulation.<sup>12</sup> But more cogent reasoning is induced by factual evidence. First, cardiac fibrosis was demonstrated in this series quite frequently—23 times in 57 cases, or in 40.4%. In a previous study<sup>13</sup> I have tried to show that cardiac fibrosis of the liver represents pathogenetically a venocapillary sclerosis consequent upon an intrahepatic venocapillary hypertension. Even when there was no frank cardiac cirrhosis, the liver showed venous congestion, usually in the advanced phases. Assuming that the average normal weight of the liver is 1,500 gm., enlargement of the liver was noted in 12 of 39 cases in which the weight was recorded, or in 30.9%, which is a further manifestation of engorgement. Second, substantiation of the portal hypertension was the finding of "congestive splenomegaly" in 22, or in 38.5%. In most instances the lesions were in the early phases, as manifested by fibrosis of the pulp, dilatation, and, exceptionally, hyperplasia of the sinusoids. In a few cases the lesions were approaching those observed in cirrhosis. That "congestive splenomegaly" may occur in cardiac disorders is well known.<sup>13</sup> I have tried to show that the lesions of "congestive splenomegaly" were the result of portal hypertension.<sup>14</sup> Pathogenetically, "congestive splenomegaly" may also be interpreted as an intrasplenic venocapillary sclerosis, com-

parable in mechanism to cardiac cirrhosis. Such spleens are almost invariably enlarged; and, as an additional support to this interpretation, it is noteworthy that the spleen was enlarged in 37 cases, or in 74% in which the weight is recorded. Third, and finally, evidence of hypertension of the systemic veins is revealed in the observation cited above, namely, that phlebosclerosis of the inferior vena cava was invariably present in congestive failure. Of the 58 cases of hepatic disorders, there were 2 in which congestive failure was associated. These two showed phlebosclerosis of the vena cava. In all the others the sclerosis was limited to the portal vein.

The causes of the congestive failure are various and are shown in Table 2.

It is evident that coronary disease and valvular disorders, particularly mitral stenosis, are by all odds the main offenders. Diabetes mellitus was a complication in nine cases, or 15.8%, but neither in congestive failure nor in hepatic disorders did it affect either the incidence or the intensity of the lesion.

It is apparent that any cardiac disorder that leads to congestive failure is potential in ultimately inducing a sclerosis of the portal vein. It is noteworthy that such a small rise of pressure within a vein is sufficient to produce a phlebosclerosis. In Whipple's series<sup>11</sup> of portal obstruction, the highest portal pressure was only 500 mm

of isotonic saline. Perhaps the veins are more susceptible because they are used to bearing lower pressures. But we again emphasize that it is the maintenance, and not the height, of the pressure that is decisive. The severest phleboscleroses, as gauged by the extent of the intimal thickening, were observed in portal obstruction, because these disorders have a longer life cycle than has congestive failure, and because of the circumstance that the pressure are probably consistently higher. The frequency of esophageal varices in cirrhosis is further testimony of the presence not only of the elevation but also of the duration of the portal hypertension. The degree of thickening also appears to be correlated with the stage of the cirrhosis, being greater in the atrophic or sclerotic than in the fatty stage. In four cases in which there was a complicating thrombosis of the portal vein, the thickening was particularly prominent, as one might expect from the superimposed vascular obstruction.

Phlebosclerosis was absent in only one case. This was in a case of early fatty cirrhosis. The reason for this absence is entirely speculative.

A word concerning the incidence of the phlebosclerosis arising in the hepatic neoplasms unassociated with cirrhosis: There were two primary hepatomas of the liver-cell type. The organs were so densely infiltrated with growths that compression of the intrahepatic branches of the portal vein was surely produced. In one case this pressure was accentuated by metastatic glandular involvement in the porta hepatis. In a case of secondary carcinoma from a primary neoplasm of the gallbladder the metastatic involvement was also very extensive. In another, a metastasizing carcinoma from the lung, there was thrombosis of the hepatic veins, which we have shown can cause portal hypertension.<sup>14</sup> In the case of primary hemangioendothelioma there was an occlusion of the portal vein.

Teleologically, just as in arteriosclerosis, the collagenous thickening of the intima may

be viewed as a phenomenon compensatory to the intravenous hypertension.

### Summary

The commonest causes of phlebosclerosis of the portal vein, as manifested by collagenous thickening of the intima, are congestive failure and portal obstruction, both intrahepatic or extrahepatic. The commonest intrahepatic obstructions are the cirrheses and extensively infiltrating malignancies, whether primary or secondary. The mechanisms and proofs whereby portal hypertension follows congestive failure are submitted. It is a matter of indifference as to origin of the congestive failure, but by all odds the most frequent causes are coronary disease and valvular disorders, especially mitral stenosis. The more intensive phleboscleroses of the portal vein are in those disorders that have a long life cycle. For this reason, they are more extensive in the portal obstructions than in congestive failure. It is the maintenance and prolongation of the portal hypertension that is the vital factor in causing phlebosclerosis rather than the degree of hypertension. Teleologically, the phlebosclerosis may be viewed as a compensatory phenomenon.

25 W. 68th St. (23).

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## News and Comment

### ANNOUNCEMENTS

**Histochemistry Registry.**—In behalf of the Histochemical Society, the editorial office of the *Journal of Histochemistry and Cytochemistry* maintains a register of positions and personnel available in the field of histochemistry. Inquiries may be directed to Dr. J. B. Longley, National Institutes of Health, Bethesda, Md. A fuller statement of the principles on which the register is operated appeared in the January, 1959, issue of the *Journal of Histochemistry and Cytochemistry*.

**Inter-Society Cytology Council.**—The Inter-Society Cytology Council will hold its annual scientific meeting at the Statler Hilton Hotel, Detroit, Nov. 19, 20, 21, 1959. Paul A. Younge, M.D., secretary, 1101 Beacon St., Brookline 46, Mass.

### PERSONAL

**Dr. Wilbur A. Thomas Goes to Albany Medical College.**—Dr. Wilbur A. Thomas, of the Department of Pathology at Washington University School of Medicine, St. Louis, has been named Professor of Pathology and Chairman of the Department at Albany Medical College, and Pathologist in Chief at the Albany Hospital. Dr. Thomas assumes his duties in Albany on July 1, 1959. He succeeds Dr. Arthur W. Wright, who retires as department chairman but will continue as Professor of Pathology and as an active member of the department.

**Major General Elbert DeCoursey Named Director of Southwest Foundation for Research and Education in San Antonio, Texas.**—Major General Elbert DeCoursey has been named Director of the Southwest Foundation for Research and Education in San Antonio, Texas. This Foundation is concerned primarily with basic research in medicine.

### DEATHS

**Dr. Joseph J. Kurtin.**—Dr. Joseph J. Kurtin, Assistant Professor of Pathology at Marquette University School of Medicine in Milwaukee, died on April 12, 1959, at the Veterans Administration Hospital, Wood, Wis.

# Experimental Hydronephrosis

H. L. SHEEHAN, M.D., and J. C. DAVIS, M.D., Liverpool, England

Certain textbooks still contain the statement that sudden complete obstruction of the ureter does not produce hydronephrosis. Strong<sup>38</sup> says that this idea stems from a misquotation of Cohnheim's article in 1880. Nearly everyone who has actually worked on the subject has found that ligation of the ureter does in fact lead to a typical progressive hydronephrosis; as early as 1904 Fabian<sup>7</sup> showed this in a large series of experiments and reviewed the extensive German literature up to that date. The present study confirms the findings of previous workers, but there are sufficient new observations to warrant putting together all the facts so that an interpretation of the pathogenesis may be attempted.

## Material and Methods

The animals used were rabbits, of a wide variety of pure and mixed breeds. They were all fully grown, ranging in weight from 2.0 to 3.6 kg., with a mean of 2.4 kg. About half of them were males. None of these factors appeared to affect the experimental results. Maatz<sup>27</sup> has shown that young animals develop hydronephrosis more rapidly than old ones; for this reason, the present study was restricted to adult rabbits.

The experiments consisted of simple ligation of the ureter about 2 cm. below the lower pole of the kidney through a midline abdominal incision. In order to avoid any possibility of spontaneous reopening of the lumen subsequently, the ureter was tied very firmly at three separate places several millimeters away from each other. At various intervals, from one day to four months later, the kidneys and ureters were examined at laparotomy under ether anesthesia and then removed for histological study. The animal was then killed.

The tissues were fixed within two or three minutes after removal. The fixative used was a saturated aqueous solution of mercury bichloride, followed 12 hours later by one day in formol-saline.

Submitted for publication Oct. 8, 1958.

Department of Pathology, University of Liverpool.

Three main groups of experiments were performed in order to assess the effect of different functional states of the kidney at the time its ureter was ligated.

1. In 12 animals the other kidney was left undisturbed so that it could take over the full burden of renal excretion when the ureter of the experimental kidney was tied.

2. In a group of four animals the contralateral kidney was removed at the same time that the ureter of the experimental kidney was ligated; these experiments were designed to give the experimental kidney the entire onus of trying to excrete urine.

3. In the third group, comprising 13 rabbits, the contralateral kidney was removed three weeks to five months before the ligation of the ureter of the experimental kidney. The purpose of this procedure was to study not only the factor of sole responsibility for attempted excretion, but also the effect of preexistent hypertrophy of the experimental kidney.

These last two groups of experiments on the influence of a preliminary unilateral nephrectomy were of necessity short-term ones, as rabbits usually can not survive more than about four days of anuria. The animals were killed one to three days after the ligation of the ureter in order to avoid natural death and thus the danger of post-mortem autolysis.

Though the pathologic changes in the three groups of experiments show some differences related to the functional condition of the kidney at the time of the ligation of the ureter, the general pattern of the lesions is basically similar. It is therefore convenient to describe the composite picture first; the variations among the groups will be detailed in the appropriate context. It must, however, be emphasized that the description of the changes after three days is based entirely on experiments on two-kidney animals.

## Macroscopic Appearances

The various changes that occur in the hydronephrotic kidney are shown diagrammatically in Figure 1.

*Animals with Intact Contralateral Kidney.*—At one day after the operation, the ureter is moderately dilated down to the site

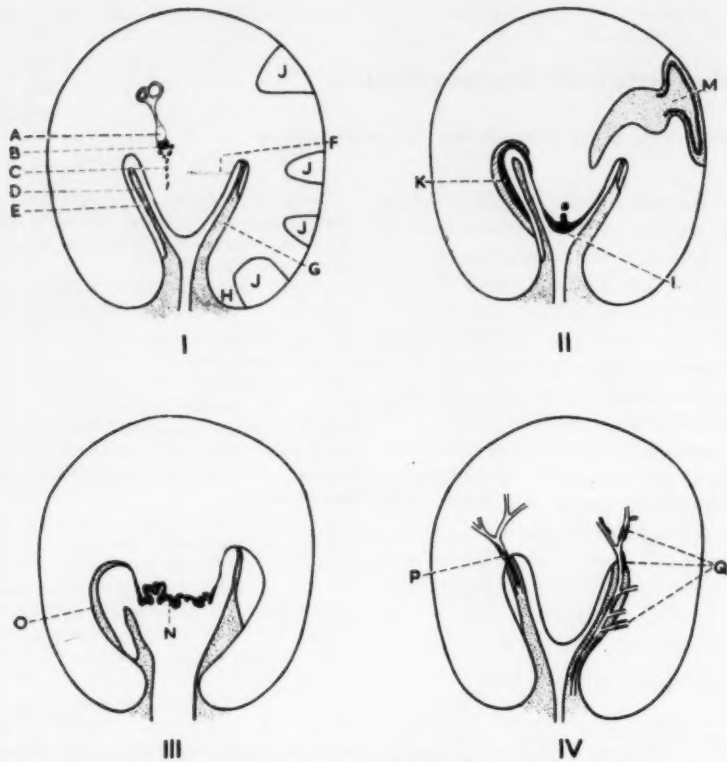


Fig. 1.—Topography of lesions produced in the kidney by ligation of ureter.

Diagram I: *A*, arcuate fibrous band; *B*, subarcuate cytolitic necrosis; *C*, line of congestion descending from arcuate band; *D*, secondary pouch of pelvis; *E*, pelvic septum (plane of section passes through the part with a free semilunar edge); *F*, shear lamina of congestion and tubular damage; *G*, pelvic septum (plane of section passes through the insertion of the vessels into the fornix and down along the subsidiary septum); *H*, parahilar cortex; *J*, wedges of cortex showing various degrees of damage, from simple congestion and colloid change up to coagulative necrosis.

Diagram II: *K*, cytolitic necrosis in peripelvic column and fornix; *L*, cytolitic necrosis of tip of pyramid; *M*, area of infarction of cortex and intermediate zone, with extension down into medulla. The site of the polymorphonuclear-leukocyte zone is indicated by a dark line.

Diagram III: *N*, stump of pyramid with necrosis in progress at surface, at about three weeks after ligating ureter; *O*, fibrous layer replacing earlier necrosis of peripelvic column.

Diagram IV: *P*, thrombosis of pelvic veins at fornix; *Q*, ruptures of internal elastic laminae of arteries.

of ligation. The kidney is enlarged, mainly as a result of the accumulation of 2 to 5 ml. of urine in the pelvis. When this urine has been allowed to escape, the kidney is nevertheless heavy; it weighs 8 to 9 gm., as compared with the normal weight of 7 to 8 gm. At two days, the ureter is markedly dilated, and the emptied kidney weighs 10 to 18 gm. (mean 15 gm.). At 3 days, the dilatation of the ureter is greater, and the kidney is even heavier, its empty weight

being 18 to 25 gm. (mean 22 gm.). Subsequently the ureter remains very large. There is a gradual dilatation of the pelvis during the next couple of weeks, but the weight of the empty kidney still remains high, at 19 to 22 gm. The enlarged kidney is easily palpable in the live animal. At two to four months, the pelvic and ureteral dilatation is very gross (Fig. 2); the parenchyma is considerably expanded and is rather tough. In an experiment continued



Fig. 2.—Dilatation of kidney and ureter two months after ligation of the ureter. The site of ligature is seen near the lower end of the photograph.  $\times 1.1$ .

for four months, the kidney itself weighed 31 gm.; its pelvis and the upper 3 cm. of the ureter contained 74 ml. of fluid.

*Animals with Only One Kidney.*—When the contralateral kidney is removed at the time that the ureter is ligated, the experimental kidney increases in weight, and its ureter dilates at about the same rate as in an animal with two kidneys. When the contralateral kidney has been removed one to five months previously, the experimental kidney is recognizably hypertrophied before the ureter is ligated; the weight at that time is presumably about 10 gm., as judged from the data of Hinman<sup>17</sup> for rabbits whose opposite kidney has been removed. After the ureter is tied, the kidney gradually becomes heavier, perhaps rather more quickly than in the other two groups; the mean weight at two to three days is 29 gm. The rate of dilatation of the ureter is similar to that in the other experiments.

*Appearances in Vivo.*—When the kidney is observed during life at the final laparotomy, the surface may be either red or, more commonly, a dusky-purple color. In the animals with only one kidney, there are often irregular, darker-purplish patches on the surface as early as one day after the

ureteral ligation, and these patches remain obvious up to the third day. Animals with two kidneys occasionally show a similar change during the first three days, but these lesions become inconspicuous and disappear after that time.

In some cases the veins in the perirenal fat or around the ureter are dilated during life, as has been noted by previous authors.<sup>12,13</sup>

A third feature is that in the animals with only one kidney some edema of the perirenal fat develops at about one day and becomes very gross at two and three days, so that the whole of the retroperitoneal tissue on that side is a gelatinous mass in which the lobules of fat lie separated from each other. This edema appears first near the hilus of the kidney, and not around the site of ligation of the ureter. In the immediate vicinity of the renal capsule, spreading out from the hilus, and sometimes reaching halfway round the kidney, there is not uncommonly a layer of greenish edematous fibrin, which in a few cases contains frank blood. Both the perirenal and the retroperitoneal edema occur fairly constantly in these animals with one kidney, irrespective of the interval since the removal of the contralateral kidney. On the other hand, in animals with a functioning contralateral kidney, the edema is slight or absent.

*Changes in the Kidney at Removal.*—When a normal kidney is removed from a living rabbit, about 1 ml. of blood escapes from the kidney by way of the cut renal vein during the first two or three seconds, and during the next minute there is a gradual ooze of about 1.5 ml. of blood and interstitial fluid from this vessel.<sup>35</sup>

The course of events is very significantly different if the hydronephrotic kidney is removed with the ligated portion of the ureter intact. When the vessels are cut, the usual amount of blood escapes from the hilus during the first half-minute and then ceases. If, now, the ureter is cut across at a distance from the kidney, a large amount of urine escapes from the ureter, and, immediately afterward, there is a second, and

quite considerable, flow of blood from the renal vein.

*Appearances on Cut Surface.*—When the excised kidney is sliced across, its cut surface shows a slightly translucent, honey-colored cortex, which appears edematous and oozes clear fluid.

At one and two days, there are often small fans of stasis-congestion in the cortex, and sometimes in the underlying intermediate zone (Fig. 1, *J*). The periphery of the fans are the purple areas which are seen on the outer surface of the kidney during life. In the animals with only one kidney, these congested areas are common and well developed; in animals which have a contralateral kidney the lesions are rare and of much slighter degree. At three days, in the animals which have had the contralateral kidney removed a few months previously, the central parts of some of the congested fans in the cortex are obviously infarcts which are undergoing dehemoglobinization.<sup>30</sup> Infarcts are not seen in the animals with two kidneys or in those in which the contralateral kidney is removed at the time of the ligation of the ureter.

When the experiments have lasted more than three days (all in animals with two kidneys), there are no areas of focal congestion in the cortex; if any had been present earlier, they have vanished.

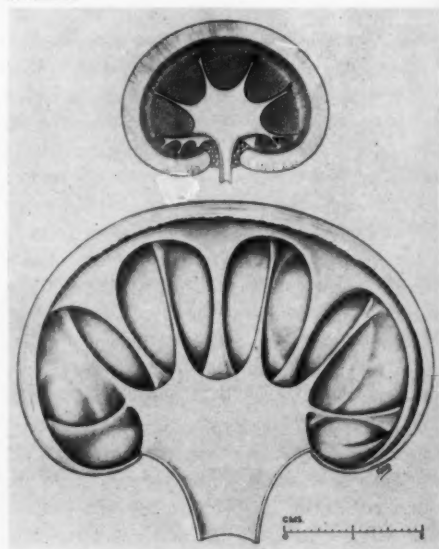
The intermediate zone is usually normal and is not clearly demarcated from the medulla. Occasionally it may show areas of stasis-congestion, and in occasional animals whose contralateral kidney has been removed a few months previously there is definite infarction of a patch of intermediate zone and of the related deep cortex or medulla.

With this exception, the medulla usually looks macroscopically normal during the first few days, though occasionally there may be a thin red line at the surface of the papilla. At about six days an important change can be seen with the naked eye, though the basic histologic lesion has in fact begun a few days earlier; the tip of the papilla disappears or is converted into a grayish slough. From this time onward

there is a progressive erosion of the stump of the papilla until, at about three to four weeks, only the base of the pyramid remains, and even this becomes flattened out in the course of the next three months. At two to four weeks the pelvis sometimes contains a mass of altered blood clot, which may be brown or yellow.

The pelvis undergoes a progressive dilatation, and, as the medulla disappears, the pelvic cavity increases correspondingly in size. At four months it is a huge oval cavity, into which project an intricate series of arches and ridges. The details of this pattern can be understood by reference to the anatomy of the normal pelvis, described elsewhere<sup>30</sup>; the nomenclature used here is explained in that paper. In the hydronephrotic kidney, the outer parts of the vascular bundles (i. e., from the level of the free semilunar edge of the pelvic septum out to the point of entry of the vessels into

Fig. 3.—Pelvic structures of normal and hydronephrotic kidneys. In the normal, the medullary pyramid has been removed to expose the septa and to show the insertion of the vascular bundles at the fornices. The peripelvic columns are seen in cross section. In the hydronephrotic kidney the medullary pyramid has disappeared. The elongated ends of the vascular bundles dip finally into the arches formed by the fusion of the peripelvic columns.



the parenchyma at the fornix) are very greatly elongated, so that they are seen as a series of prominent high ridges projecting into the cavity (Fig. 3). The bases of these ridges consist of the "subsidiary septa," which remain intact and are wider and thicker than normal. The main septa are thick but are not greatly stretched, so that their free semilunar edges remain relatively close to the hilus. The peripelvic columns are greatly elongated and widened, and form rather flattened ridges between the subsidiary septa, and separated from these by wide and moderately deep grooves. At their outer ends these columns fan out and join with their neighbors, producing a series of very large rounded arches with recesses under the arch. The grooves at the side of the peripelvic columns continue outward beneath these rounded arches and extend into the recesses there. The ridges formed by the main vascular bundles and the related subsidiary septa run outward between these grooves and end by dipping under the apex of the arches into the recesses.

### Comment

The present findings indicate quite clearly that the dilatation of the pelvis produces a partial obstruction of the main venous outflow from the kidney. This is shown by the fact that, after the kidney is removed, much of the blood in the kidney is unable to escape until the pressure in the pelvis and ureter has been released.

An observation of similar type was made by Ghoreyeb,<sup>10</sup> who tied the ureter and observed that some days later there was an impediment to artificial perfusion of the kidney. He showed that during the first week this impediment was due to the dilatation of the pelvis, since it disappeared if the distended ureter was opened and the intrapelvic pressure was thus allowed to fall. The existence of such a mechanical obstruction has been shown in short experiments by other workers. Lucas<sup>26</sup> noted that in the perfused kidney a rapid raising of the pressure in the pelvis impairs the outflow

through the renal vein. Maatz and Krüger<sup>28</sup> investigated the same point in the kidney of the living dog; they found that when the renal pelvis was distended, there was a considerable reduction in blood flow through the renal vein, as measured by a stromuhr. These experimental observations seem to be applicable to the continuing blood flow through the hydronephrotic kidney. Direct evidence on this point was obtained by Levy et al.,<sup>24</sup> who found in living dogs that the flow through the vein was considerably reduced on the third and fourth days after tying the ureter. A beautiful demonstration of the slowing of the circulation in the acutely hydronephrotic kidney has been given by Herdman and Jaco,<sup>16</sup> using serial angiography.

### Generalized Lesions in the Cortex

The histologic changes in the parenchyma of the cortex fall into two main types, generalized and focal. The present section deals with the first of these. The changes to be considered are distributed fairly generally throughout the cortex, albeit in a somewhat patchy manner. They correspond to the naked-eye appearances of a rather edematous cortex in the early stages, followed in the next few weeks by the gradual development of a fibrous atrophy.

The terminology applied to these lesions is that which has been used elsewhere in a series of papers on experimental renal ischemia.<sup>35</sup>

*Glomeruli.*—Significant lesions are not found in the glomeruli during the first two months; in particular, there is no dilatation of the capsular space and no "lacework" appearance of the tufts.

*Proximal Convoluted Tubules.*—There is an early but transient dilatation of these segments, followed by atrophy several days later.

During the first day the proximal tubules in the outer half of the cortex undergo some dilatation (Fig. 4), which is very variable in degree and distribution but tends to be most marked in radiating fans. These dilated tubules possess a brush-border, which

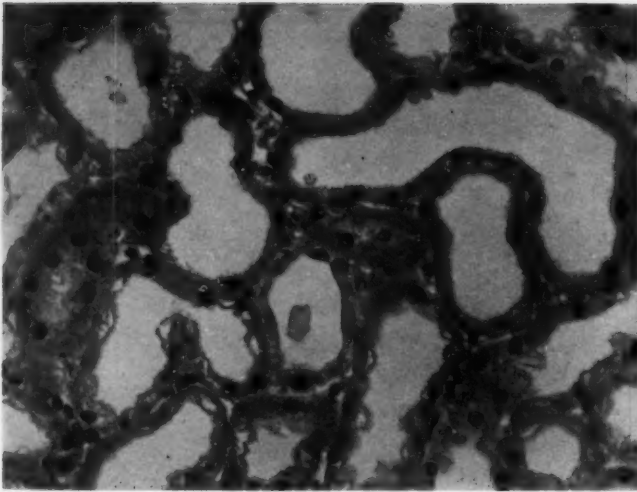


Fig. 4.—Area of gross dilatation of proximal tubules in outer cortex. One day after ligating ureter. Hematoxylin-eosin stain;  $\times 340$ .

is remarkably prominent for the rabbit. Their cytoplasm is usually solid. In only one case were a few watery vacuoles seen in the cytoplasm of these tubules; this is possibly a mild grade of the "vacuolar nephropathy" which Kulka et al.<sup>22</sup> observed in rabbits following ligation of the ureter.

At two and three days the tubules in the outer half of the cortex are less dilated, and in large areas have reverted to their normal size. Mitoses, both typical and atypical, begin on the second day and are common on the third day; this wave of mitotic activity has been recorded by Strong<sup>28</sup> and Herlant.<sup>16</sup> From the fifth to the seventh days the tubules show little or no remaining dilatation, and mitoses are very infrequent.

From the seventh day onward there is a progressive atrophy of the proximal tubules, with some thickening and wrinkling of the basement membrane and a gradual diminution of the tubular lumen. This atrophy first affects the tubules in the outer half of the cortex and then slowly involves those nearer the medulla. At two months many of the tubules are reduced to solid narrow cords of cells, but it is a striking feature that small patches of almost normal tubules with eosinophilic cytoplasm remain in the deep part of the cortex, and may persist even as long as four months.

These changes affect most of the proximal tubules in the kidney, but the cortex around the hilar tunnel appears to be relatively exempt, and may continue practically normal even as late as two months. This is probably related to the fact that the parahilar cortex drains into collateral vessels just outside the hilus (Fig. 1, *H*).

*Narrow Loops of Henle.*—There is no significant change during the first two days. On the third day, the cells are often moderately swollen, and a variable number of mitoses are present. These mitoses are seen up to about the fifth day. From one to two weeks the epithelium tends to be flattened, so that the loops are a little dilated, and at two weeks small globules of protein material are sometimes present in the lumen.

At one and at two months many of the narrow loops have disappeared; the remainder are usually represented only by a column of cells inside the thickened basement membrane, and the lumen is lost.

*Broad Ascending Limbs and Distal Convolute Tubules.*—From one day onward, the broad ascending limbs, both in the intermediate zone and in the medullary rays, and the distal convolute tubules undergo a progressive dilatation. This develops more quickly in the one-kidney animals. The lumen is usually empty, though it may con-

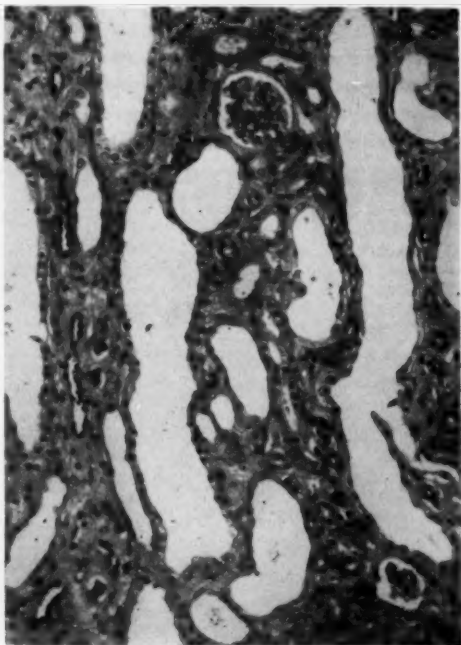
tain slight, wispy casts. On the second day the dilatation is very obvious, and the epithelium becomes flattened and rather basophilic. At the same time, normal or distorted mitoses begin to occur in it. These two features lend an appearance of recent regeneration to the epithelium, though there has been no preceding phase of necrosis. Subsequently the epithelium becomes even more flattened, but the basophilic change fades away. Mitoses are not seen after about the fifth day. From one week onward many of the tubules are dilated and tortuous, particularly in the medullary rays. After the first two or three weeks some of these dilated tubules atrophy and disappear, but the remaining ones continue to increase in diameter and tortuosity (Fig. 5), and their epithelium becomes extremely flat.

Parallel with these various changes, a special type of deposit gradually accumulates in the lumen of some of the tubules. At about three days small globules of hyaline protein appear, and, from five days onward, these globules are tinged with brown and

have a rather crystalline appearance, so that they are somewhat similar to the casts in a hemoglobinuria kidney. They do not show free iron with the ferrocyanide test. During the next few weeks these globule-casts gradually increase. At two months, they are a prominent feature, and are present in most parts of the kidney, with the exception of the relatively well-preserved parenchyma in the parahilar region. Strong<sup>38</sup> maintains that these casts are formed by the fusion of red corpuscles. However, no blood has been seen at any stage in these tubules in the present series, and, though the globules admittedly have a superficial resemblance to red corpuscles, their tinctorial reactions and the variation in their sizes prove that they are really quite different.

*Collecting Tubules.*—The generalized lesions in this segment are not as clear-cut as in the remainder of the nephron; but, nevertheless, a broad outline of the changes here can be drawn. At two days many of the collecting tubules are slightly or moderately dilated; their epithelium is flat and basophilic

Fig. 5.—Dilatation of distal and collecting tubules in cortex, with atrophy of proximal tubules. Two months after ligating ureter. Hematoxylin-eosin stain;  $\times 120$ .



and has occasional mitoses. At three to seven days the dilatation is greater, particularly in the intermediate zone. Mitoses are common at the third day, and some are still present at seven days; thereafter they are absent or very rare. From two weeks to two months, these tubules become difficult to differentiate in the confused mixture of atrophic and greatly dilated tubules. Occasionally some of them contain brown granular precipitate at that time.

*Interstitial Tissue.*—During the second day, a slight intertubular edema may be present, either diffusely or patchily in the cortex. Sometimes this becomes very marked around the capsules of the glomeruli (Fig. 6), separating them from the neighboring tubules. On the 3d day some mitoses are seen in the interstitial cells, and occasionally are very numerous; they may still be found as late as 14 days. This proliferation gives a recognizable increase in the number of interstitial cells by the fifth day. Fine fibrils appear between these cells at about the seventh day, and thereafter the interstitial tissue becomes more fibrous, particularly in the patches where the tubules are atrophic. At this time, also, there may be an increase in the collagen and elastic tissue of the adventitia around the larger

arteries in the deep cortex, as was emphasized by Fabian.<sup>7</sup>

It is important to note that in none of these cases have lesions been observed which show the slightest resemblance to tubulorrhexis, a lesion which, when present in human material, is easily identifiable in histologic sections.

*Late Stages.*—The kidney at four months has the cortex and the remnants of the intermediate zone and medulla stretched out to a thin shell. Histologically, the remains of the cortical parenchyma are widely separated by loose fibrous tissue. As a result, the glomeruli appear scanty in any one area of the parenchyma, though this is manifestly due to the great spreading out of the thin layer of cortex. The glomeruli tend to be rather flattened parallel to the surface of the kidney (Fig. 7). Most of the tubules which remain have been reduced to thin cords of cells, but rare proximal tubules with eosinophilic epithelium can still be identified; this long survival of fairly normal tubules has been noted by Lee-Brown.<sup>23</sup> In a number of tubules, possibly the remains of distal tubules, the epithelium contains a golden granular pigment, which may be due to the taking up of the cast material which was present in these tubules at two weeks.

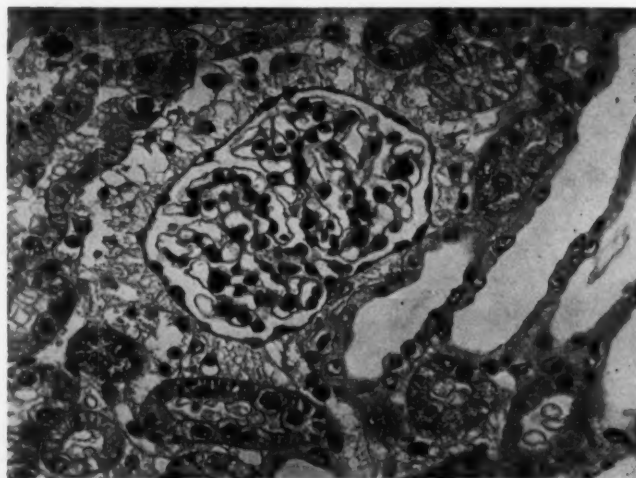
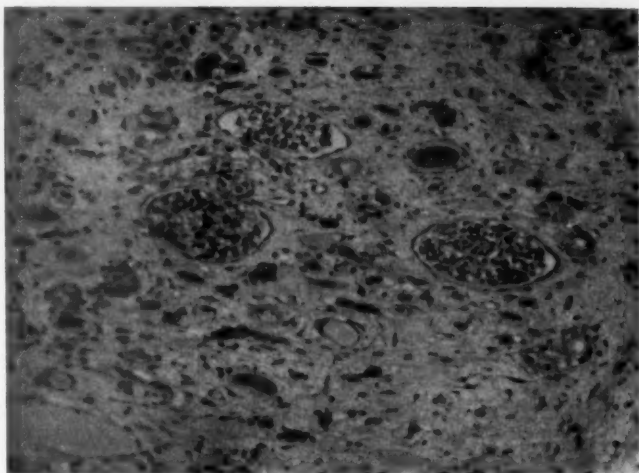


Fig. 6.—Interstitial edema around glomerulus. Two days after ligating ureter. Hematoxylin-eosin stain;  $\times 340$ .

Fig. 7.—Atrophic cortex in prolonged hydronephrosis, showing flattening of glomeruli and survival of a few proximal tubules. Four months after ligating ureter. Hematoxylin-eosin stain;  $\times 120$ .



### Comment

Two possible explanations of these diffuse lesions in the cortex will be considered here: obstruction of outflow from the nephrons, and pressure by the dilated pelvis.

**Obstruction of Outflow from the Nephrons.**—It is generally accepted that urine continues to be secreted by the nephrons. The "obstruction theory" envisages that the urine cannot escape freely from them because the raised pressure in the pelvis blocks the outflow from the ducts of Bellini, and this leads to atrophy of the parenchyma.

Any lesion which is to be explained as due purely to a general obstruction of all the nephrons should be present evenly throughout the cortex. The only lesion which approaches this criterion is the minor dilatation of the proximal convoluted tubules during the first two or three days, but even this is never quite uniform and is commonly absent from the parahilar cortex.

In the late stages, after several weeks of hydronephrosis, there are still a few proximal tubules with almost normal histologic appearance scattered in a background of condensed stroma from which the other proximal tubules have vanished. This is, of course, not conclusive proof that obstruction is not operative, since it is well known that

different units in an organ do not always respond uniformly to a standard insult.

The dilatation of the broad ascending limbs and distal convoluted tubules is much more patchy in distribution. It is very difficult to give a satisfactory explanation for this lesion. Any hypothesis of increased internal pressure comes up against the difficulty that the narrow loops and the medullary collecting tubules do not dilate, nor, at the other end of the nephron, do the capsular spaces of the glomeruli. The fact that the dilatation of this segment is a progressive one during the first two or three weeks raises the alternative possibility that it might be a specific type of "hypertrophy." There is certainly some evidence that this part of the nephron continues to function: In the early stages some of these tubules become filled with colloid material, and in the later stages coarsely granular casts accumulate in the lumen. Strong,<sup>38</sup> as a result of his microdissection studies, concludes that these wide tubules are in fact cysts cut off from the nephron above and below.

A further difficulty in the obstruction theory is that, though the pressure in the lumen of the tubule is presumably somewhat raised, this increase does not necessarily imply an arrest of flow down the tubule; one must take into account the large, but

unknown, amount of "pyelovenous back-flow" that may allow escape of urine from the pelvis. Thus, it is not really permissible to infer that when the ureter is ligated, there is in fact a serious degree of obstruction to the outflow of fluid from the nephrons themselves.

*Pressure by the Dilated Pelvis.*—The second mechanism by which pressure from the pelvis might produce obstruction of tubules is a local one. It may be postulated that short segments of certain nephrons are flattened, either by direct squeezing or by stretching as a result of the dilatation of the pelvis. This could occur at any place where the intermediate zone and outer medulla lie in contact with the pelvis or its secondary pouches. This explanation has been advocated by Maatz<sup>27</sup> and Strong.<sup>38</sup> The latter author states that the greatest damage is inflicted on the tubules in two special regions of the cortex; (a) at the convex border near the base of the pyramid, and (b) near the hilus. Our own findings are not in agreement with this pattern. The distortion of the architecture of the nephrons certainly tends to be in wedges, but these wedges are distributed very irregularly throughout the cortex. Furthermore, though the parenchyma some millimeters away from the hilus is often very badly damaged, the immediately parahilar cortex is often the least affected part of the kidney and is sometimes almost completely normal histologically. This parahilar region is the extreme end of Strong's "lateral cortex" and, on the local-pressure hypothesis, the nephrons here ought to be the most vulnerable because their loops are exposed to the most stretching or kinking.

#### Focal Lesions in the Cortex

The lesions described in this section correspond to the clearly defined fans of congestion in the cortex and the outer part of the intermediate zone, which were described in the macroscopic appearances of the cut surface of the kidney. It is convenient to consider them under four headings according to the severity of the damage. All the

types originate fairly soon after the ureter is ligated, and are seen at one to three days.

*Congestion and Colloid Change.*—This is the mildest type of lesion. The areas may occur anywhere in the cortex, including its deeper half. They have a well-marked radial pattern, which is due to congestion of the intertubular capillaries in the cortical tissue between the medullary rays, the latter being little, if at all, congested.

Microscopically these congested areas have a characteristic group of features. The glomeruli contain little blood. The capillaries between the convoluted tubules are filled with red corpuscles, and there is some extravasation of corpuscles into the interstitial tissue. The extravasation is particularly marked in the tissue immediately surrounding the glomeruli, as has been noted previously by Corbett<sup>5</sup>; this is the region where edema is prone to occur in the non-congested areas of the cortex. The proximal convoluted tubules are not dilated. Their epithelium is rather high, and may have some degree of hydropic degeneration or, more commonly, numerous hyaline droplets, which vary in size and are occasionally very big indeed. Some of these proximal tubules show "colloid degeneration." The broad ascending limbs, the distal convoluted tubules, and the cortical parts of the collecting tubules in these areas are not dilated, but they are filled with a very solid-looking and strongly eosinophilic colloid. These changes may be combined in different ways. In general, it appears that the presence of colloid in the lower nephron as the sole lesion is evidence of the mildest vascular disturbance.

This grade of lesion is quite well developed at one day and continues unchanged to three days; it has not been seen after that time, possibly because the changes are reversible.

*Small Areas of Necrosis of Proximal Tubules.*—Many of the changes in this grade are similar to those in the previous one. Congestion and hemorrhage are present; there is colloid in the lumen of the lower

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nephron, and the changes in some of the proximal convoluted tubules are no severer than in the first grade. There are, however, certain additional lesions. Some proximal tubules show "colloid necrosis." Others have undergone ordinary "coagulative necrosis," which often involves several neighboring coils but may affect only small groups of cells or isolated cells in the tubule.

In general, this coagulative necrosis occurs in patches scattered in the outer quarter of the cortex, though there is commonly a zone of live tubules immediately beneath the renal capsule. The less severe type of lesion (the colloid degeneration and colloid necrosis) occurs as a zone around these areas of coagulative necrosis.

*Large Areas of Necrosis of Proximal Tubules.*—This grade appears to be merely a severer and more extensive version of the previous one. Coagulative necrosis is seen in all the proximal tubules of one or more large areas of the cortex, usually in its outer half. There are no appearances to suggest that these tubules have undergone any previous hyaline-droplet or colloid change. The distal convoluted tubules in the area sometimes contain colloid; but the more severely damaged ones have an empty lumen, and occasionally their epithelium is necrosed. Very rarely, there may be necrosis of a lobule of a glomerular tuft. The intertubular

capillaries are usually not significantly congested, and their endothelial nuclei appear normal.

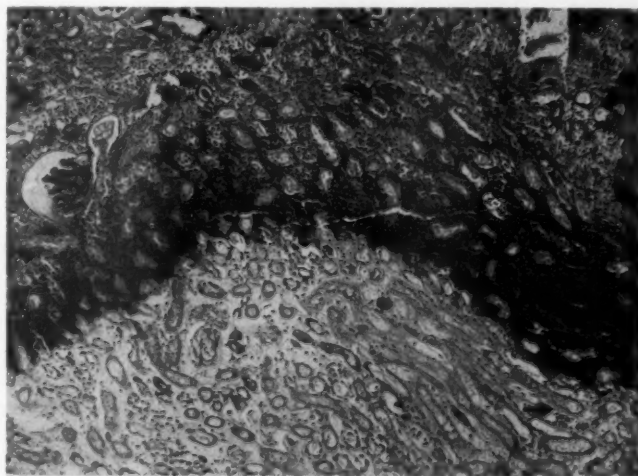
These large areas of tubular necrosis are usually surrounded by broad zones of parenchyma showing less severe lesions; these are of the type described in the two previous sections.

As early as one day after the ureter has been tied, the necrosis is quite obvious and the nuclei have disappeared. At two and three days a few tubules may show karyorrhexis or incomplete karyolysis: It is difficult to decide whether this indicates recent death of these tubules, or whether it is merely due to a very slow rate of necrosis change.

Regenerative changes appear in some of the necrosed tubules by the third day, with typical spreading in of basophilic epithelium and numerous mitoses. After about a week this grade of lesion can no longer be distinguished with any degree of certainty, because the regenerated tubules have very similar appearances to those of neighboring tubules which are undergoing atrophy.

*Infarction.*—The severest grade of lesion is the least common; it is true infarction (Fig. 8). When this occurs, it usually involves an area of the deep cortex and the adjacent parts of the intermediate zone and of the outer medulla. There may also be

Fig. 8.—Edge of infarct at junction of cortex and intermediate zone. The polymorphonuclear-leukocyte zone is well developed. Three days after ligating ureter in a one-kidney animal. Hematoxylin-eosin stain;  $\times 65$ .



infarction of a patch of subcapsular cortex, overlying the deep infarct and connected to it by a narrow waist (Fig. 1, *M*). In rare cases, tiny wedge-shaped infarcts are present in the outer cortex alone. The histologic appearances of the fully developed infarcts indicate that the lesion probably begins within a few hours after the ureter has been ligated. In the cases where the outer surface of the infarct is separated from the renal capsule by a broad band of non-infarcted cortex, the intralobular veins which run from this live tissue down into the infarct are thrombosed at the site where they traverse the peripheral dead zone. The arteries which enter the infarct at its deep aspect remain normal in the very congested partial-survival zone, but are dead and filled with occlusive thrombus at the polymorphic zone; further on they show mural thrombosis, exactly as in the center dead area of infarcts produced by obstruction of the main renal artery.<sup>35</sup>

The deeper part of the infarct of the intermediate zone extends down as a nar-

rower "tail" into the medulla. If the infarct is situated opposite the base of the pyramid, this extension runs a straight course into the center of the pyramid. On the other hand, when the main mass of the infarct lies in a lateral part of the kidney, the extension bends around the fornix to reach the side of the pyramid (Fig. 9). It may end here, or it may continue into one of the patches of necrosis of the pyramid, which will be described later.

Helmholz and Field<sup>14</sup> observed "infarcts" in 3 out of 20 kidneys of dogs in which they had tied the ureter for 18 hours or longer. It is not clear whether these were infarcts in the true sense or merely wedges of congestion with necrosis of some tubules.

*Effect of Removal of the Contralateral Kidney.*—Table 1 shows the incidence of these focal lesions in animals examined during the first week after the ureter was ligated. It will be seen that there are no significant differences between the animals in the first two series: those in which the contralateral kidney was intact, and those in which it was removed at the time of ligation of the ureter of the experimental kidney. In contrast, the animals whose contralateral kidney had been removed several weeks previously show a great increase in the incidence and severity of the lesions. Areas of tubular necrosis are present in almost every case, and true infarcts of the cortex are confined entirely to this series.

Fig. 9.—Red infarction of cortex and intermediate zone, extending down to involve half the pyramid on the right side. Three days after ligating ureter of a one-kidney animal.  $\times 2.3$ .



TABLE 1.—Influence of Opposite Kidney on Occurrence of Overtly Ischemic Lesions in Cortex During First Week After Tying Ureter

	Not Removed	Removed at Time of Tying Ureter	Removed Several Weeks Before Tying Ureter
Infarcts or large areas of necrosis	0	0	5
Small areas of necrosis	2	1	6
Only colloid changes or congestion	1	2	1
No overtly ischemic lesions	5	1	1

### Comment

It is convenient to consider the fourth of these lesions first, and to work backward from this.

The infarcts are clearly ischemic in origin.

The large and the small areas of necrosis of proximal tubules have the same pattern as the infarcts. Histologically, all their characters can be reproduced experimentally by transient ischemia, and there can be little doubt that this is their etiology in the hydronephrotic kidney.

The least severe focal lesions are the congested patches or radiating fans in various parts of the cortex which show colloid casts and hyaline droplets. These may also be accepted as ischemic, for two reasons. First, the same changes can be reproduced experimentally by an episode of ischemia insufficient to kill the proximal tubules. Second, though these areas commonly occur quite independently, all the grosser lesions (such as infarcts or patches of necrosis of tubules) are surrounded by a broad zone of parenchyma showing the same colloid casts, hyaline droplets, and intertubular congestion. As these grosser lesions are ischemic, it seems probable that the zone surrounding them has suffered a less severe degree of ischemia.

At this point, we may refer back to two of the relatively diffuse lesions discussed in the previous section. These are the dilatation of the broad ascending limbs and distal convoluted tubules, and the more gradual development of atrophy of proximal tubules. There is certainly a case for regarding them as due to slighter but long-continued ischemia: (a) They tend to have the same patchy distribution in the cortex as the obviously ischemic lesions; (b) they commonly, though not invariably, spare the immediately parahilar cortex, which, as has been mentioned earlier, appears to have a venous drainage different from that of the remainder of the cortex; (c) a moderate degree of experimentally produced ischemia of the kidney causes almost identical lesions in these segments.<sup>35</sup> Thus, these changes in

the tubules, which were not satisfactorily explicable by the theory of obstruction of nephrons, would fall into place as the lower end of the series of ischemic lesions, of which infarction is the upper end.

If it be accepted that the cortical lesions are ischemic in origin, certain further inferences must be drawn. The ischemia is permanent only in the rare sites where infarction occurs. On the other hand, practically the whole cortex remains alive and continues to secrete some urine into the pelvis; so it must be accepted that the ischemia is incomplete, or only transient, throughout most of the kidney.

The question arises how such a vascular disturbance might be produced. The circulation might be impaired or arrested either in the arteries or in the veins. The infarcts are the obvious place where evidence may be sought on this point, but the search is often fruitless. Commonly there is no gross change in the main vessels leading to or from an infarct, apart from the standard necrosis and secondary thrombosis of all the vessels where they traverse the margin zones. In a single case there was necrosis and thrombosis of an artery at the tip of the infarct; this is the only example in which the arterial supply was certainly obstructed. On the other hand, in a number of cases the possibility arises that obstruction of the vein may have been responsible. In these kidneys the infarcts in the superficial cortex were accompanied by thrombosis of the veins at the pelvic fornix directly beneath them, though at some distance from them. This subject will be discussed later.

There are other reasons for believing that the cortical lesions of hydronephrosis are due to partial venous obstruction. The most significant evidence comes from a study of the effect of narrowing of the main renal vein.<sup>35</sup> This experimental procedure reproduces every change that is seen in the cortex in experimental hydronephrosis, from infarction or wedges of necrosis of proximal tubules down to the minor grades, such as colloid in distal tubules, droplets in proximal

tubules, and congestion and diapedesis in the intertubular tissue. Furthermore, it is followed by dilatation of distal tubules and gradual atrophy of the proximal tubules.

One important point of difference between infarcts due to venous obstruction and those due to ordinary arterial obstruction is that the former are always surrounded by a zone of colloid and droplet change, whereas this zone is usually absent around arterial infarcts. In the hydronephrotic kidney the infarcts always have a peripheral zone similar to that around infarcts produced by narrowing the main renal vein. This is in keeping with the view that the infarcts in the hydronephrotic kidney are caused by venous obstruction and thus that the other focal lesions in the cortex have the same origin.

#### Necrosis of Medullary Pyramid

Although it is generally recognized that the pyramid disappears in experimental hydronephrosis, many authors tacitly assume that this is the result of a gradual atrophy. In fact, it is produced by necrosis, which begins in the superficial parts of the pyramid within the first three days and progresses steadily during the next month, so that finally the entire pyramid is eroded away, right to its base. The true nature of this process has been recognized by a number of workers who have studied the effects of experimental ligation of the ureter.<sup>7,14,30,37</sup>

*Appearances at One to Two Days.*—The necrosis is not very easy to discern with the naked eye until about the end of the first week, but histologically the initial stages can be found sometimes as early as one or two days, and almost always at three days. In those experiments in which the contralateral kidney has been removed several weeks previously, the development of necrosis in the pyramid is accelerated. As is seen from Table 2, the lesion is common on the first day in such animals.

In some cases the necrosis involves the tip of the papilla, cutting transversely across all the ducts of Bellini; in some cases it spares the tip and involves only a layer on

TABLE 2.—Effect of Removal of Contralateral Kidney on Incidence of Necrosis of Medullary Pyramid After Tying Ureter

Days After Tying Ureter	Contralateral Kidney Intact	Contralateral Kidney Removed at Time of Tying Ureter	Contralateral Kidney Removed One to Five Mo. Before Tying Ureter
1	0/2	--	2/2
2	0/1	0/2	1/2
3	1/2	2/2	9/9
Over 3	8/8	--	--

one side of the pyramid; in other cases both sites are affected. The necrosis of the side of the pyramid may be continuous, with a long band of necrosis running around the fornix and down the peripelvic column; this will be described later.

The lesion at this stage does not involve the actual surface of the pyramid. The area of necrosis may lie immediately under the pelvic epithelium, or it may be separated from this epithelium by a layer of living tubules and interstitial tissue, in which there are normally staining red corpuscles (Fig. 10). The pelvic epithelium covering the surface of the papilla usually remains alive and apparently healthy; the cells may be rather tall, with the free border of each cell projecting in a verrucose manner.

The affected area shows a special histologic appearance, which will be referred to here as "cytolytic necrosis." There is a rapid destruction and disappearance of the epithelium of the collecting tubules and loops of Henle, but the basement membranes remain intact (Fig. 11). Even in the early stages, at about 24 hours, the tubules usually have no trace of epithelium remaining; their lumen is empty or shows a little granular debris or fibrin. Sometimes, however, the dead collecting tubules contain a number of dissociated rounded epithelial cells, which can have remarkably normal nuclei. Occasionally a few red corpuscles may be present. The interstitial tissue becomes slightly eosinophilic.

In the neighborhood of the areas of full necrosis, there are usually patches of partial necrosis which show a peculiar differential

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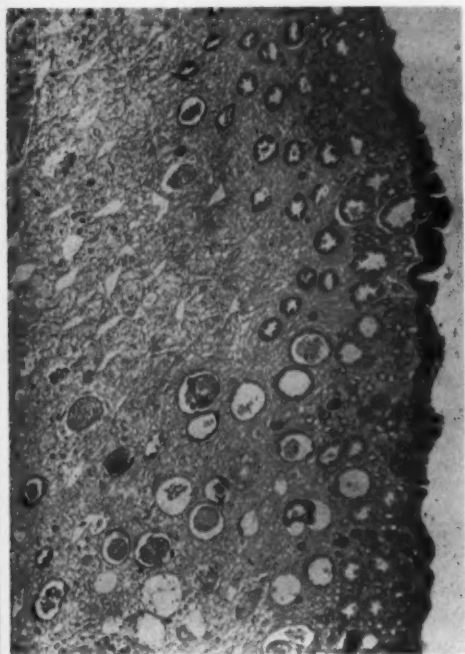


Fig. 10.—Cytolytic necrosis of much of the pyramid, with survival of a layer beneath the pelvic surface. Five days after ligating ureter. Hematoxylin-eosin stain;  $\times 65$ .

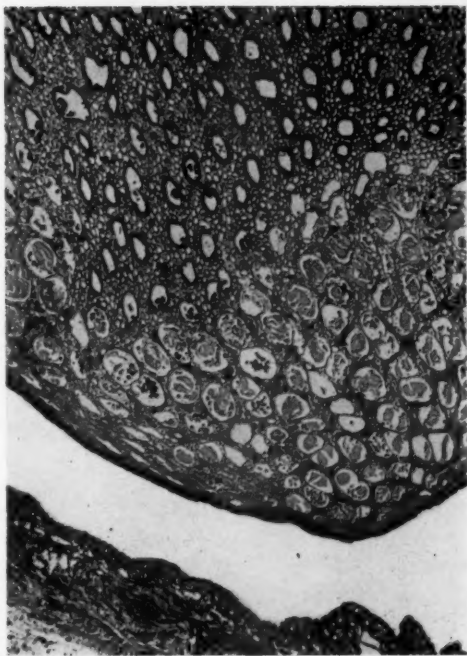


Fig. 11.—Cytolytic necrosis near tip of pyramid, with survival of pelvic epithelium over it. Four days after ligating ureter. Hematoxylin-eosin stain;  $\times 70$ .

involvement of tubules. Sometimes the nuclei of the collecting tubules appear almost normal, but the narrow loops of Henle have gross pyknosis. In other places the collecting tubules show extensive pyknosis, but a number of the narrow loops survive and may contain colloid casts.

*Appearances at Three Days.*—When the necrosis in the pyramid is about three days old, practically all the nuclei in the area have disappeared, and the tissue has a faded "ghost appearance," with an open meshwork consisting mainly of basement membranes. Very rarely even the basement membranes disappear, leaving a space which is filled with necrotic debris and communicates with the cavity of the pelvis. There is never a polymorphonuclear-cell zone; in the occasional kidneys where these cells are seen, they are scanty and confined to the area of necrosis, and nothing suggestive of an ascending pyelonephritis has been observed.

The inner margin of the necrotic area is sharp, and where the necrotic area cuts across a duct of Bellini, this duct opens out

as a broad estuary into the dead tissue (Fig. 12). The epithelium appears to be spreading out from the lower end of the duct, in an attempt to cover the junction between the live and the dead tissue. The live collecting tubules near the margin of the necrosis, or even for about 0.5 mm. above it, show numerous mitoses, and their epithelium is often piled up to form a thick, irregular layer, particularly on the side nearest the area of necrosis (Fig. 12). There is also much mitotic activity in the narrow loops in the same area. Some of the collecting tubules closely adjacent to the edge of the necrotic tissue contain rounded-off cells, which have desquamated into the lumen.

The pelvic epithelium over the surface of the necrosed area usually remains intact at this stage, despite the fact that the necrosis of the underlying parenchyma may be rather extensive; occasionally it may disappear in a small patch overlying the central parts of the necrosed tissue. In some cases the epithelium that remains is stretched and

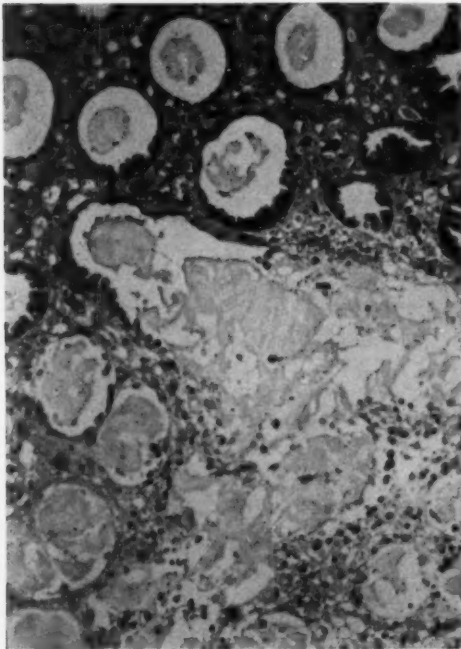


Fig. 12.—Cytolytic necrosis of tip of pyramid, cutting across a duct of Bellini. There is polypoid proliferation of the epithelium of surviving collecting tubules on the side next to the necrosis. Six days after ligating ureter. Hematoxylin-eosin stain;  $\times 150$ .

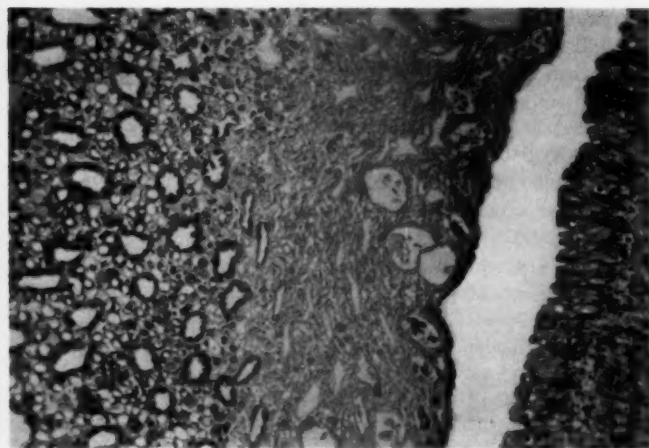


Fig. 13.—Cytolytic necrosis at side of pyramid. The dead tissue is covered by a flat layer of epithelium in the lower part, but is denuded in the upper part. Seven days after ligating ureter. Hematoxylin-eosin stain;  $\times 120$ .

thin (Fig. 13), but in other cases it is proliferating and is thrown into marked papillary projections. This epithelium sometimes contains large round masses of eosinophilic hyaline material.

**Later Stages.**—During the period from five days to two months, two opposing processes are at work: a progressive necrosis, which ultimately destroys the pyramid, and a reactive attempt to repair the damage.

After about the third day the tissue at the surface of the pyramid begins to disappear, either by a slow crumbling or as a massive slough, and presumably becomes lost in the debris and altered blood in the pelvis. At the raw surface there is a layer of fresh necrosis, and, in the live tissue beneath this, small areas of early necrosis (Fig. 1, *L*) are seen; the changes correspond in miniature to those which have been described earlier. This process leads to a gradual destruction of the pyramid, both by a nibbling away of the surface and by an excavation extending inward from the original site of the papilla toward the center of the pyramid.

There is threefold evidence that this necrosis is a serially progressive one. First, these areas of fresh necrosis are seen in all cases up to the end of the first month; second, the pyramid disappears little by little from its tip down to its base during this period; third, in the remaining stump of the pyramid the live medullary tissue abuts

sharply on the necrotic area, without any intervening zone suggestive of "atrophy."

The destruction of the pyramid would leave a raw surface over its stump were it not for the reactive changes which proceed *pari passu* with the necrosis. Epithelium spreads, both from the neighboring live parts of the pelvis and from the mouths of the tubules, to cover the denuded surface. A small amount of loose fibroblastic tissue may be present beneath this new epithelium. These areas are destined to undergo further cycles of necrosis and imperfect healing, so that the appearance at about three or four weeks is that of a short pyramid whose apex is hollowed out into a bay into which some of the collecting tubules open, with patches of further necrosis beneath the surface (Fig. 1, *N*). At four months the pyramid has been entirely destroyed; on looking into the pelvis of the gross specimen, all that is seen at the site of the pyramid is a long narrow strip of whitish tissue. The surface is now fully epithelized and has a thin layer of fibrous tissue beneath it; no further necrosis is observed at this stage.

### Comment

The most satisfactory explanation of the necrosis of the pyramid is that it is due to ischemia. It has been concluded in the previous section that when an acute hydronephrosis is produced by ligation of the

ureter, the focal lesions in the cortex are due to an impairment of the circulation there. This circulatory disturbance presumably involves the medulla, as well as the cortex. The tip of the pyramid, being at the extremity of the medullary circulation, would presumably be the most vulnerable area. In addition, there is a local factor affecting the circulation through the pyramid: The raised intrapelvic pressure will tend to compress the capillaries there, for reasons which are discussed below. A relatively minor increase of pressure in the pelvis might be the factor which determines the continuance or cessation of the blood flow in the pyramid. A possible additional factor is that the enlargement of the pelvis and the damage to the septa will prevent the contractions of the pelvic muscle from assisting the blood flow through the medulla.

This explanation in terms of a pressure ischemia is based on the interpretation of two points: the cytolytic necrosis and the hydrodynamics of the pelvis.

*Cytolytic Necrosis.*—This term denotes a special histologic appearance of dead tissue which is very different from that of ordinary coagulative necrosis. The characteristic feature is that all the dead cells disappear rapidly, leaving a fairly open network of basement membranes and of interstitial collagen or reticulin, with no trace of cell debris. The capillaries may or may not contain some red corpuscles; but, if corpuscles are present, they appear normal and do not show any dehemoglobinization.

It is important not to interpret the histologic picture as representing the actual conditions during life. In particular, the presence of red corpuscles in the capillaries must not be taken to indicate that there was a blood flow there during life. The appearances can best be explained in the following way:

The initial disturbance is a raising of the pressure in this particular area because of the distention of the pelvis. As a result, the capillaries are flattened completely and carry no blood flow. This ischemia leads to death

of all the cells in the area in the course of a few hours.

The next stage is the continuation of the pressure, but now on dead tissue. The necrotic epithelium of the tubules undergoes rapid lysis, probably to fluid or semifluid consistency, and is extruded from the area. If it were possible to examine the medulla microscopically in the live animal, and to see the conditions as they actually are at this stage, the picture would be one of the basement membranes of the tubules compressed together to form a layer in which there would be practically no remaining lumen, no cells or cell debris, and no blood corpuscles.

The final change is a postmortem artifact which produces the histologic appearance actually seen in sections. When the animal is killed and the renal pelvis is opened, the pressure on the dead tissue is released. The previously compressed stroma now tends to open out as a result of its natural elasticity. In addition, the fluid in the surrounding kidney tissue flows into the dead area and expands the stromal network. A specific example of this inflow of fluid is that, if there happens to be much blood in the neighboring live parts of the kidney, this blood flows back into the capillaries of the dead area; this is particularly the case if the ureter is opened before the vein. A similar course of events accounts for the fact that in some cases desquamated epithelial cells are present in the tubules of the necrotic area. During life these cells were in the partly damaged tubules outside the margin of the necrotic area, and had rounded off and fallen free into the lumen. After the kidney is removed, these cells are washed down into the dead tubules of the necrotic area.

This explanation fits all the facts, and, though it is scarcely susceptible of direct proof, there is other evidence to support it. First, in the present experiments cytolytic necrosis has been found not only in the medullary pyramid but also in two other sites: the peripelvic columns and the

arcuate fibrous bands. Both of these are places where it appears clear on mechanical grounds that there must be a marked local rise of pressure. Second, in infarction of the medulla produced by ischemia or vinylamine poisoning<sup>35</sup> the pressure in the pelvis is not raised, and cytolytic necrosis of the dead medulla does not occur. Instead, the tissue seems to undergo coagulative necrosis, but this is always masked by a gross congestion of all the capillaries there. This is a true picture of the condition *in vivo*, because, if the lesion is 18 hours old, the red corpuscles in the area are undergoing dehemoglobinization, indicating that they have been stationary in an area of ischemia for that time.<sup>35</sup> A third piece of evidence is that, in the areas of cytolytic necrosis which occur in the peripelvic columns and beneath the arcuate fibrous bands, there is never any regeneration of tubules or other sign of repair; the stroma merely condenses to a fibrous mass. This is in accord with the concept that the area where the cytolytic necrosis has occurred continues to be exposed to the pressure for a long time afterward. The necrosis of the medullary pyramid cannot be used to check this point, because the area is destroyed by ulceration before repair could occur.

We regard cytolytic necrosis as a basic process in general pathology. It appears that necrosis is "cytolytic" instead of "coagulative" in any area where dead tissue is immediately exposed to continuous high pressure. Thus, it is well seen in hydronephrosis but is not confined to that condition. As a simple example, if an artery is killed by transient ischemia and blood is then allowed to flow through the vessel at ordinary arterial pressure, the dead media undergoes very rapid cytolytic necrosis and disappears.<sup>35</sup>

*Mechanical Conditions in the Hydronephrotic Pelvis.*—As has just been said, it appears that cytolytic necrosis in the pyramid and the other two sites is due to localized pressure effects on the tissue there. The localization of these effects in the case of the pyramid and the peripelvic columns

is susceptible of the following physical explanation. Though the raised fluid pressure in the pelvis is distributed evenly throughout the cavity, the actual mechanical stresses will fall particularly on the summits of any salients that project into it. This is because a cavity of irregular shape is "unstable," so that any increase of pressure in it produces forces tending to the adoption of the spherical shape. Thus, where the inner surface is concave, it is subjected to tension; and where it is convex, it is subjected to pressure. This latter condition applies to the salients of the pyramid and the peripelvic columns.

*Course of the Necrosis.*—It is difficult to explain why the necrosis of the pyramid processes in a gradual, though rather irregular, manner during the three or four months after the ureter is tied, so that finally the whole pyramid is destroyed. Two factors may be relevant. First, the pelvis is undergoing a progressive enlargement, so that the causes of necrosis which operated in the first few days may well continue in action during the later stages. Second, the cortex atrophies gradually after the first few weeks, and, though this does not give rise to any gross changes in the arteries or glomeruli, the intertubular capillary bed of the entire cortex must certainly be distorted. This might possibly lead to some further impairment of the flow to the medulla.

### Necrosis in the Peripelvic Columns

Necrosis which is in many ways analogous to that in the medullary pyramid occurs along the summits of the ridges of the peripelvic columns. The lesion commonly extends around the fornix and becomes confluent with an area of necrosis at the side of the pyramid (Fig. 1, K). The condition is occasionally well developed as early as one day, and is very common from three days onward.

*Early Stages.*—Microscopically, the more extensive lesions show three regions. The area lying along the summit of the column, and thus nearest the pelvis, undergoes cyto-

lytic necrosis almost identical with that which occurs in the medullary pyramid; the tissue consists only of basement membranes, with no trace of epithelial debris in the lumen. The pelvic epithelium over the necrosed area survives. Beneath the area of cytolytic necrosis there is a broad and rather congested zone where the tubules show ordinary coagulative necrosis and are filled with epithelial debris, sometimes entangled in fibrin. Finally, in the outermost zone (i. e., nearest the cortex) there is no necrosis, but a variable number of the tubules contain colloid casts. Most of these seem to be broad ascending limbs, a segment which is particularly prone to undergo this change. When the patches of necrosis are small, the surface area of cytolytic necrosis is absent, and the lesion consists only of a zone of coagulative necrosis at the summit of the column with a zone of colloid casts beneath it.

Around the fornix the lesion is seen as a thin band close beneath the pelvic surface; commonly it is only about half-a-dozen tubules deep. In this band, there is a pecu-

liar mixture of dead tubules filled with debris and of live tubules filled with colloid; congestion is usually conspicuous here.

Where the lesion extends down the side of the pyramid, it runs deep to the surface. It is sometimes covered only by a layer of live pelvic epithelium, but there may also be a layer of partly damaged tubules just beneath this epithelium, with colloid casts in some of the narrow loops. The main mass of the full necrosis is of the standard cytolytic type.

*Later Stages.*—The healing stages of these lesions follow a fairly regular pattern. The areas of cytolytic necrosis under the pelvic epithelium probably shrivel to insignificant amounts of stroma. In the middle zone of coagulative necrosis there is some regeneration at about the third day; dead tubules are being relined by new epithelium, and in places this is spreading over the debris in the lumen. Later on, nearly all these regenerated tubules disappear, and this region is gradually replaced by fibroblastic tissue in which only occasional tubules are present (Figs. 1, O, and 14).



Fig. 14.—Healed stage of necrosis of peripelvic column, showing condensed stroma with very few remaining tubules. Two months after ligating ureter. Hematoxylin-eosin stain;  $\times 120$ .

## EXPERIMENTAL HYDRONEPHROSIS

The columns can still be seen with the naked eye several months later as very obvious anatomical structures. The pelvic epithelium continues permanently intact over the surface. There is never any massive ulceration of the type seen in the pyramid.

### Comment

The changes in the peripelvic columns are very similar to those in the pyramid, and are probably due also to a pressure ischemia produced by the same physical factors that were invoked in the case of the pyramid.

The main lesion is the cytolytic necrosis of the summit of the column, i. e., the apex of the salient which projects into the corresponding secondary pouch of the pelvis. Further out, where the pressure is less intense, the necrosis is of ordinary coagulative type. The tendency of the lesion to extend around the fornix and down the pyramid may possibly be due to occlusion of the intertubular capillaries which follow this course; the occlusion might be generalized

or at only one point, such as in the peripelvic column.

No adequate reason can be given for the fact that the necrosis of the peripelvic columns reaches its full extent within two or three days and then heals, whereas the pyramid undergoes progressive necrotic ulceration for many weeks.

### Necrosis Beneath Arcuate Fibrous Bands

This lesion is a cytolytic necrosis which affects the tubules of the intermediate zone in a very small area just beneath the arcuate fibrous band (Fig. 1, *B*). The tubules here lose their epithelium completely, so that they are represented only by basement membranes enclosing open empty spaces; the connective tissue is pale and eosinophilic and is devoid of nuclei (Fig. 15). Some of the vessels in this region may contain red corpuscles, but most are empty. Occasionally a band of intensely congested vasa recta runs down from the arcuate fibrous band into the medulla, where it ends in the pyramid; this is an exaggeration of a minor

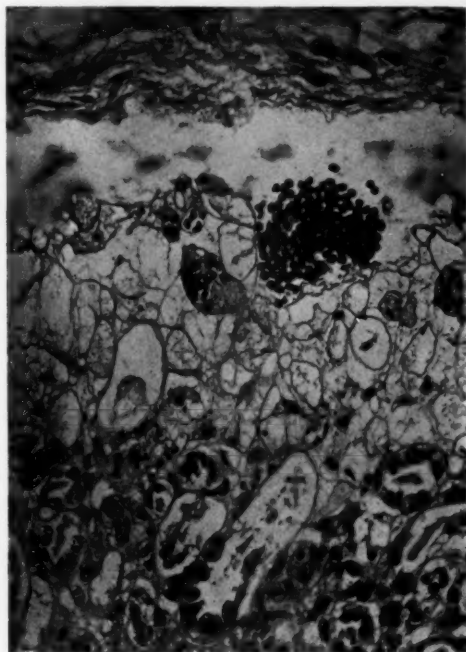


Fig. 15.—Area of cytolytic necrosis of tubules under arcuate fibrous band. Two days after ligating ureter. Hematoxylin-eosin stain;  $\times 340$ .

condition seen in normal kidneys. The necrosis is sometimes very well developed as early as two days, but there is no recognizable reaction at that stage. At six days a doubtful trace of interstitial fibrosis may be present, but no regeneration of tubular epithelium can be observed. The later stages cannot be identified; it seems probable that the necrosed mass shrinks into a small knot of condensed stroma and interstitial tissue, which loses its identity by fusing with the undersurface of the arcuate fibrous band.

#### Comment

Though this lesion is not common, and is never big, it is important because it provides an excellent example of cytolytic necrosis and thus throws light on the mechanical conditions in the hydronephrotic kidney. While the animal is alive, this area is almost certainly subjected to a very localized pressure. The dilatation of the pelvis will push the medulla out toward the cortex, and at the same time the tension on the septal vessels will pull the arcuate bands toward

the hilus. The tissue just beneath the relatively unyielding arcuate bands is thus sandwiched between two opposing forces, and the capillaries here will be obliterated by the pressure. In addition, the pressure applied to the cells is directional and thus could crush them in a way that uniform pressure cannot do. This may explain why the ischemic area, though it is so small, does not survive as a result of diffusion.

The dead cells undergo cytolytic necrosis because they remain subject to localized pressure, just as in the case of the medullary pyramid and the peripelvic columns.

#### The Shear Lamina

This is a minor lesion which is seen in transverse sections of the kidney as a band of altered parenchyma and blood vessels running across the base of the pyramid (Fig. 1, *F*). The band starts at one side of the pyramid a little below the fornix and runs inward for a variable distance, commonly about one-third or halfway across the pyramid, though exceptionally it may reach

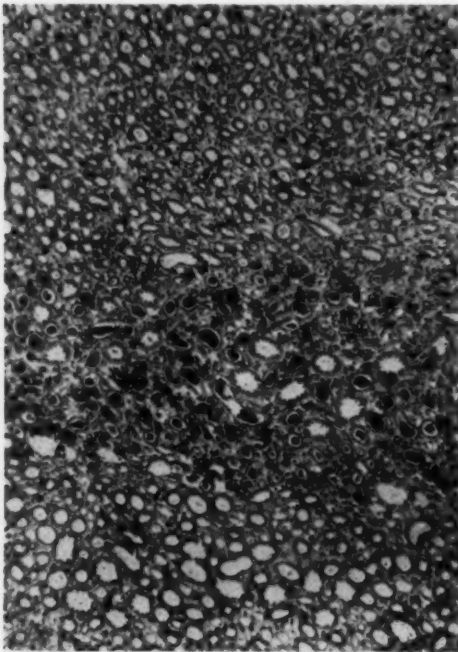


Fig. 16.—Shear lamina at base of pyramid. The vessels are congested, and many of the tubules contain colloid casts. Four days after ligating ureter. Hematoxylin-eosin stain;  $\times 70$ .

almost to the other side. Occasionally it is bilateral; and, if so, it is usually very much better developed on one side than the other. Its thickness ranges from  $30\mu$  to  $300\mu$ , with an average of about  $100\mu$ .

Histologically the shear lamina is characterized by congestion of the vasa recta, filling of the narrow loops of Henle with colloid casts, and the presence of fibrin and red corpuscles in some of the collecting tubules (Fig. 16). When the lesion is severe, a number of the tubules are necrotic, and there is hemorrhage in the interstitial tissue. In rare cases the part nearest the fornix is very broad and continues out as a large area of necrosis beneath the surface of the pyramid, indistinguishable in type from the necrosis of the papilla.

Although this lesion is seen in sections as a thin band, it is clear that in fact it represents a lamina projecting into the base of the pyramid. The layer of tissue on each side of this lamina appears very healthy and is not congested.

A shear lamina is seen in most, but not all, of the cases at one to five days. It may be a very prominent feature in the section, or it may be so slight that it escapes notice unless specially looked for.

### Comment

The shear lamina is a most difficult lesion to understand. The appearance is very similar to the deeper zones of necrosis of the peripelvic columns, and this similarity suggests that it also is due to some type of pressure ischemia.

There are two outstanding problems. The first is why the ischemia should be confined to this thin lamina of tissue traversing the base of the pyramid on one side. The only theory we can offer depends on the asymmetry of the two faces of the pyramid, related to the difference in size of the two septa. The asymmetry might cause the pyramid to be bent to one side when the intrapelvic pressure is raised, and thus give rise to a line of shearing stress near its base.

The second problem is to explain how the tissue on the papillary side of the shear

lamina remains alive; the lesion might be expected to arrest the blood supply of all the tissue beyond it. One is forced to the *ad hoc* explanation that blood passes through those parts of the base of the pyramid which are not involved in the lamina, and then circulates through anastomosing capillaries to supply the part of the pyramid below the lamina.

### Changes in Wall of the Pelvis

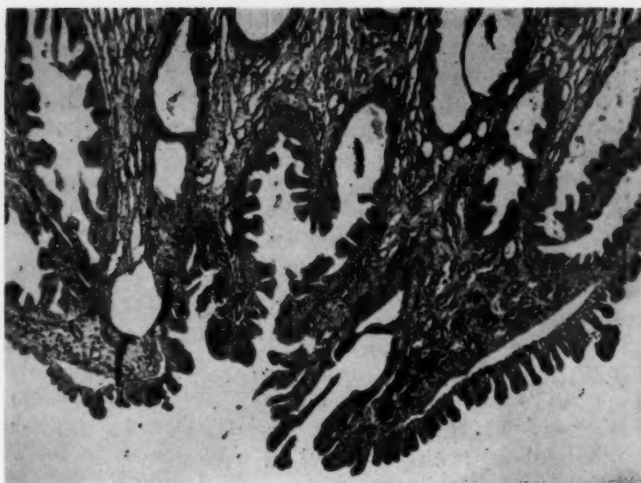
*Pelvic Epithelium.*—Occasionally on the second and third days numerous red corpuscles are seen spreading out among the apparently normal cells of the parietal epithelium, particularly near the opening of the ureter. Sometimes these form tiny blood blisters under the surface of the epithelium. In other cases, as has been noted by Morrison,<sup>20</sup> there may be similar blisters containing granular material, which was presumably fluid before fixation. These lesions heal without leaving any recognizable traces.

Proliferative changes are seen in the pelvic epithelium in the course of the first two weeks. On the first day there are occasional mitoses, and these become more frequent during the next few days. At this stage the epithelium is often thickened and thrown up into small papillae; the cells here may contain numerous large hyaline droplets. Similar papillary outgrowths occur at the lower ends of the ducts of Bellini in the early stages before the tip of the pyramid is necrosed (Fig. 17). The mitoses and the thickenings of the epithelium are seen up to two weeks, but after this time the epithelium settles down to a rather flattened type.

*Fissures in the Wall of the Pelvis.*—In places on the surface of the septum, particularly near its attachments at the fornix, there are sometimes gaps in the epithelium and the underlying muscle. These may be seen as early as one day after ligating the ureter, as has been noted by Helmholtz and Field.<sup>14</sup>

We are uncertain as to whether these gaps are true lesions or artifacts. It is possible that they may be true lesions which

Fig. 17.—Papillary proliferation of the pelvic epithelium over the tip of the pyramid and also in the ducts of Bellini. In this particular specimen the tip of the pyramid was not yet involved by necrosis. Three days after ligating ureter. Hematoxylin-eosin stain;  $\times 65$ .



develop in vivo, either as a result of bursting of the septum by the accumulation of edema fluid within it or possibly as a result of tearing by longitudinal stretching. Reactive changes are sometimes seen in the tissues beneath the tear at about three days, but this does not prove conclusively that the tear occurred in vivo; similar changes may be occurring anywhere in the septum at this time, and thus what is seen may be merely an accidental exposure of such reaction by a tear at the time the kidney is sliced. Furthermore, we have not seen any appearances suggestive of healed tears in cases of longer duration.

The other possibility to consider is that the tears might be artifacts produced during the slicing of the kidney before fixation. Similar tears are not seen in normal kidneys, but this is not an adequate control; after ligation of the ureter the septa are very edematous and thus may be unduly friable. To check this point, three additional experiments were performed in which the ureter was tied, and, three days later, the kidney was removed and was fixed entire before it was sliced. No tears of the surface of the septa were found. However, this negative result is not of high significance, because tears are seen in only about half the cases in which the hydronephrotic kidney is sliced before fixation.

At about three to seven days very small foci of necrosis may be seen in the parenchyma underlying the fornix just beside the base of the pyramid; these sometimes contain a few disintegrating polymorphonuclear leukocytes, so that they are acceptable as true lesions. Occasionally a small split in the wall of the pelvis is present at the site of this necrosis. A number of previous investigators, who have observed these splits in the fornix, accept them as true in vivo lesions and as exaggerations of the intercellular gaps which normally allow pyelovenous backflow (Fuchs<sup>9</sup>). However, it is again difficult to exclude the possibility that the splits are artifacts produced during the slicing of the kidney; they have not been seen in kidneys fixed entire before slicing. This region is certainly a weak point in the pelvis, and tears can be produced there very easily by the injection of 1 or 2 ml. of fluid up the ureter of a normal kidney which has been removed from the body.

#### Changes in the Pelvic Septa

As early as one day after tying the ureter there is gross edema and usually some diffuse hemorrhage in the fatty tissue of the septum (Fig. 1, E and G), and polymorphonuclear leukocytes may be present in ill-defined patches there. Changes of this kind have previously been described by

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Helmholz and Field.<sup>14</sup> On the second day the edema is usually more marked, and, in addition, there may be irregular areas of strongly eosinophilic fibrinoid material in the fat. These areas are particularly well developed around certain arteries and also in a layer underneath the muscle on the inner surface of the septum. By the third day there is a diffuse proliferation of fibroblasts, apparently beginning in the adventitia of some of the arteries. These cells invade the fibrinoid material and remove it during the next couple of days. The fibroblastic reaction then subsides, leaving the pelvic fat slightly fibrosed; a few small foci of lymphocytes may be seen here even as late as two months.

A peculiar disturbance occurs at about two days in the muscle layer on the inner surface of the septum. Its peripheral part near the fornix remains normal, but in the part opposite the tip of the papilla gross edema develops. The muscle cells here become widely separated from one another and thus their histological appearance is very peculiar; but they can easily be identified by following them along to the normal muscle layer. On the third day these separated muscle cells show numerous mitoses and are certainly proliferating rapidly. During the next few days the muscle coat slowly reverts toward its previous appearance, though it now appears hypertrophied, and the normal division into a layer of longitudinal and a layer of transverse fibers is exaggerated.

The edema fluid in the septum distends certain thin-walled vessels there, which have many valves and are presumably lymphatics. It then passes through the hilar tunnel and turns outward in the renal capsule and in the perirenal fat for some distance from the hilus. This exudate around the kidney contains much fibrin during the first three days. Soon afterward it becomes organized by fibroblasts, and has been converted to a coat of fibrous tissue by about two or three weeks.

Small hyaline, rounded masses, sometimes concentrically laminated, are usually present

in the pelvic cavity, especially near the fornices, and can be recognized with the naked eye as a sludge of white material there. They are also seen histologically in the fatty tissue of the edematous septa; and, when there is a spread of fluid from the hilus around the capsule of the kidney, they are often carried in large numbers to this site.

A special example of the spread of edema fluid from the septum is sometimes seen in the lower ends of the secondary pouches of the pelvis, where these approach the hilus (Fig. 1, *D*). Here the fluid spreads beneath the pelvic epithelium, raising this and its associated connective tissue from the renal parenchyma beneath. This heals later to form a rather thick layer of connective tissue.

## Comment

The edema and hemorrhage in the fatty and muscular tissue of the septa may be caused in two ways. First, when the ureter is obstructed, there is a pyelovenous backflow,<sup>18,19,32</sup> and, as one stage of this, a "pyelointerstitial" backflow must be postulated. It is possible that much of the edema and hemorrhage in the septa is due to the large volumes of urine "back-flowing" into them from the pelvis and causing local irritation. A second explanation is that, if the vein is obstructed at the hilar tunnel, this would lead to a passive engorgement of the septa, and thus to the exudation of fluid there. In whatever way this change in the septa is produced, the fluid in them must be under considerable pressure, because it oozes out through the partially obstructed hilar tunnel into the capsule and the tissues outside the kidney, and produces a low-grade inflammatory reaction there.

The perirenal edema is a prominent feature only in the animals with one kidney. Two possible explanations may be suggested. First, the amount of urine secreted by the hydronephrotic kidney is probably greater in the one-kidney animal than in the two-kidney animal, and thus there is more pyelointerstitial backflow. Second, the ani-

mal with two kidneys is capable of excreting fluid by its normal kidney and therefore could absorb any edema fluid which escaped from the hydronephrotic kidney.

The subsequent fate of the perirenal fibrinous edema is of some interest. If it should happen to occur in an animal which had a functioning contralateral kidney and could thus survive, the local reaction might give rise to a "thick-walled perinephric cyst." In fact, Girgensohn<sup>11</sup> has observed such a cyst after ligation of one ureter in the rabbit. The condition is of importance because similar perinephric cysts are occasionally found in human beings; Girgensohn discusses the whole question and reviews the literature.

We can offer no very satisfactory explanation why the peculiar edematous disruption of the muscle of the septum should be localized to the level between the hilus and the tip of the pyramid. It occurs in the early stages, and presumably must be ascribed to the rapid dilatation of the pelvis. The fact that there is no corresponding change in the muscle in the more peripheral part of the septum or in the ureter suggests that, under normal physiologic conditions, these parts may be more accustomed to transient dilatation, whereas the region where the damage occurs has a relatively constant diameter

under ordinary circumstances until it is suddenly expanded by the hydronephrosis. The final hypertrophy of the muscle of the whole septum is probably a process of adaptation to the dilatation and increased pressure in the pelvis.

### Lesions of Arteries

#### Rupture of Internal Elastic Lamina

One of the most striking lesions in the acutely hydronephrotic kidney is the development of multiple ruptures of the internal elastic lamina of the larger arteries of the pelvis (Fig. 1, Q), the other parts of the arterial wall at the site of these ruptures apparently being undamaged.

The ruptures occur at intervals along the course of the artery, and the parts of the artery between the ruptures show no abnormality. The tear in the elastic lamina is transverse or oblique (Figs. 18, 19, 20, 21, and 22), and its edges are rather sharp. The remaining parts of the lamina have an astonishingly normal appearance, and nearly always retain their corrugations up to the cleanly broken end, though exceptionally a portion retracts and forms a folded and distorted layer (Fig. 23). In longitudinal sections the remaining parts may thus appear slightly thickened. If an artery is cut in transverse section through the center of

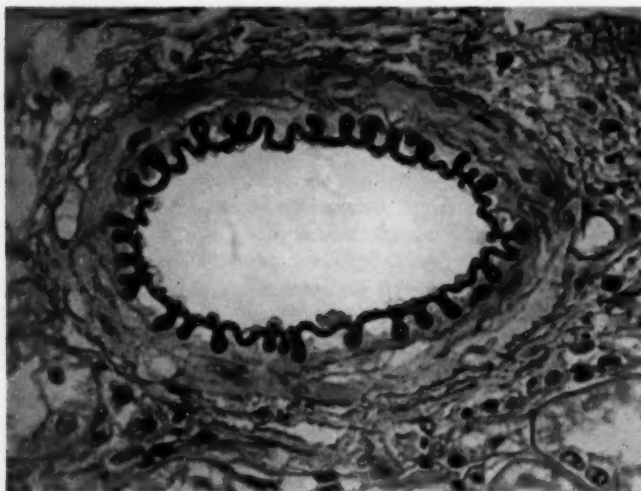


Fig. 18. — Intralobular artery from kidney of normal rabbit, showing intact internal elastic lamina. Weigert stain;  $\times 340$ .

Fig. 19.—Small gap in internal elastic lamina. Three days after ligating ureter. Weigert stain;  $\times 340$ .

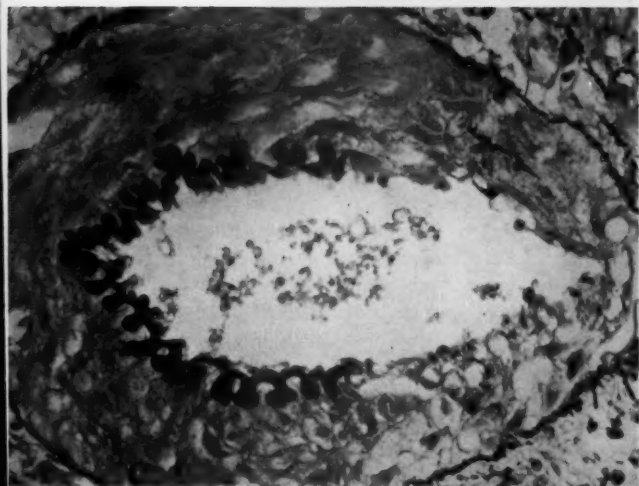
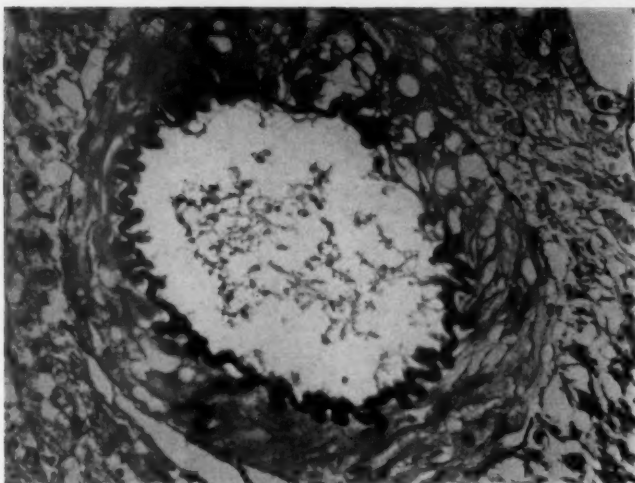
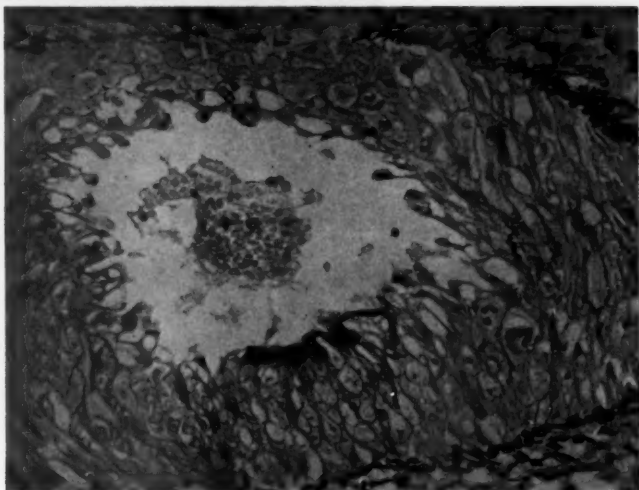


Fig. 20.—Large gap in internal elastic lamina. Three days after ligating ureter. Weigert stain;  $\times 340$ .

Fig. 21.—Very extensive loss of internal elastic lamina. Three days after ligating ureter. Weigert stain;  $\times 340$ .



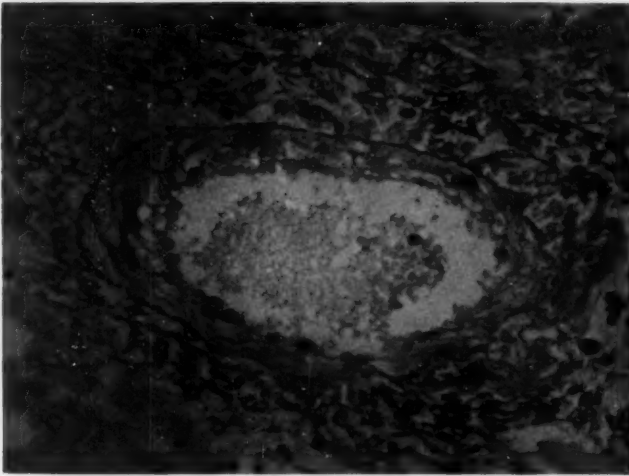


Fig. 22.—Total disappearance of internal elastic lamina. Three days after ligating ureter. Weigert stain;  $\times 340$ .

a complete rupture, no trace of the elastic lamina can be seen (Fig. 22). Such a vessel appears relatively normal in hemalum-eosin preparations, and the lesion can easily be overlooked unless careful examination is made of a section stained by Weigert's elastin method.

The lesion nearly always obeys an all-or-none law. Only occasionally is an incomplete version seen, with a portion of the lamina stretched out and greatly attenuated but not ruptured.

Rupture of the internal elastic lamina occurs in various parts of the arterial tree. Commonly, the large arteries which run in the two main connective tissue septa of the pelvis are involved at some part between the hilar tunnel and the fornix. The major branches from these, which run out in the subsidiary septa or at the fornix, are also commonly affected. Less frequently, ruptures may be seen in the arcuate arteries in the region of the deep cortex and outer intermediate zone. No lesions of this type

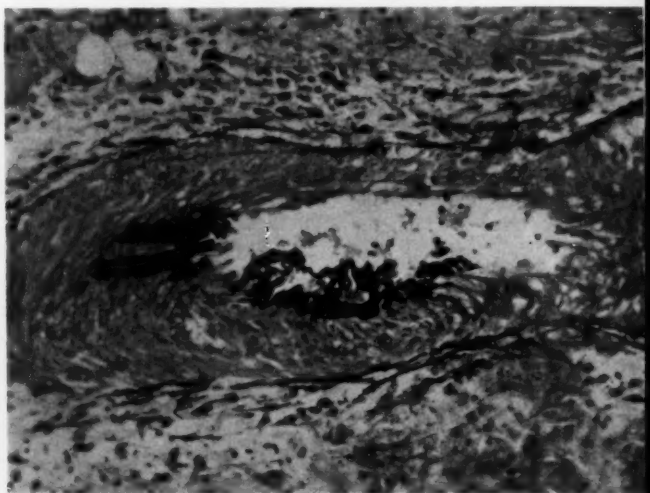
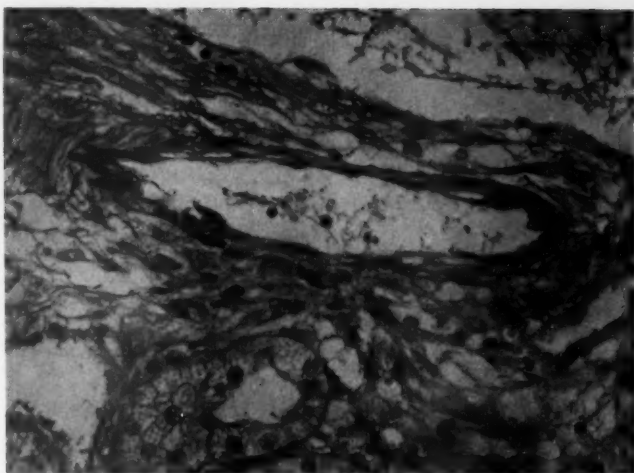


Fig. 23.—Rupture of internal elastic lamina, showing retraction to a tangled mass. Three days after ligating ureter. Weigert stain;  $\times 340$ .

Fig. 24.— Rupture of internal elastic lamina, with extreme thinning of media. Three days after ligating ureter. Weigert stain;  $\times 340$ .



have been seen in the intralobular arteries or their branches.

The number of arteries affected is also very variable; sometimes in a single transverse section of the kidney only two or three examples can be found, sometimes about a dozen. If one of the large arteries in the main septa has ruptures of the elastic lamina, its branches are commonly affected also.

Usually the media shows no gross lesion at the site of these elastic tears, though it sometimes seems slightly edematous. Occasionally, however, it is greatly thinned or

almost disappears (Figs. 24 and 25). This medial damage may be diffuse, or it may be sharply localized to the segment where the internal elastic lamina is ruptured; in this latter case, the artery assumes a D-shape in transverse section (Fig. 26). This occurs particularly in the neighborhood of "elastic cushions," structures which will be discussed later.

We have commented elsewhere<sup>35</sup> on the surprising fact that, though ischemic mediolysis (i. e., destruction of the media of small arteries in the kidney by a transient obstruction of the main renal artery) com-

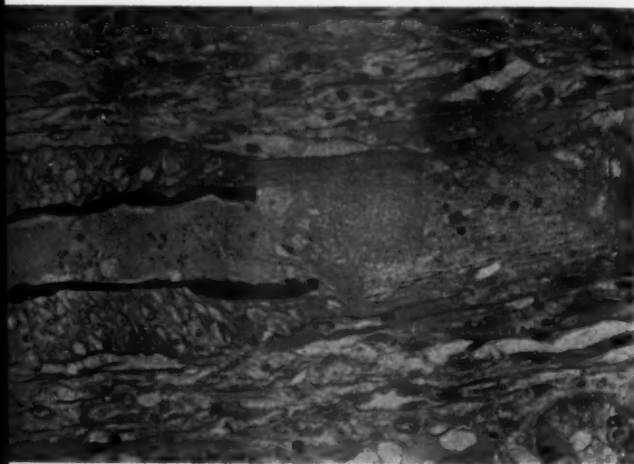


Fig. 25.— Rupture of internal elastic lamina, with hyaline change and disintegration of media. There is some terminal thrombosis. Two days after ligating ureter. Weigert stain;  $\times 340$ .

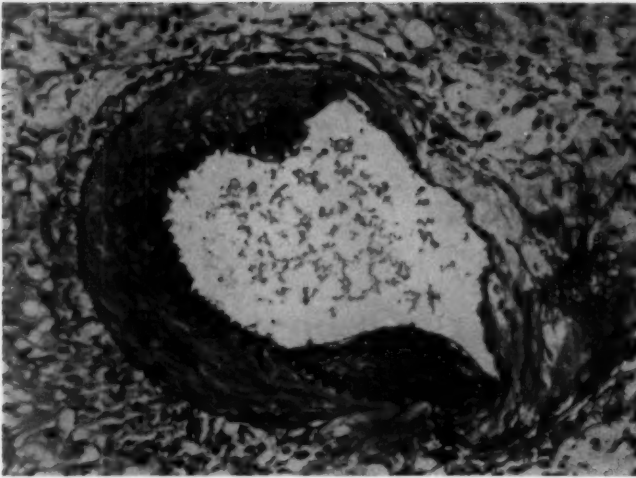


Fig. 26. — Rupture of internal elastic lamina and disruption of underlying media, giving a D-shaped artery. Two preexistent elastic cushions are seen near the ends of the tear. Three days after ligating ureter. Weigert stain;  $\times 150$ .

monly produces a moderate dilatation of the arteries, it does not give rise to aneurysm formation or to bursting of these vessels. The internal elastic lamina remains intact in that condition, and thus it might perhaps be considered that this was the structure which prevented the vessel from bursting. The arteries in the hydronephrotic kidney present the reverse condition. The elastic lamina itself is absent at the site of the rupture, but the media usually remains intact there; the lumen is of normal caliber (Fig. 27) or shows only a slight fusiform

bulging (Fig. 28). It might be postulated that in these hydronephrotic kidneys it was the media which preserved the integrity of the vessel. However, as has just been mentioned, there are occasional arteries in which the internal elastic lamina has ruptured and, in addition, the media has almost disappeared; but, nevertheless, these arteries retain their continuity.

At the site of the rupture of the elastic lamina, the intima almost invariably appears undamaged. We have found only a single case of thrombosis; in one artery which

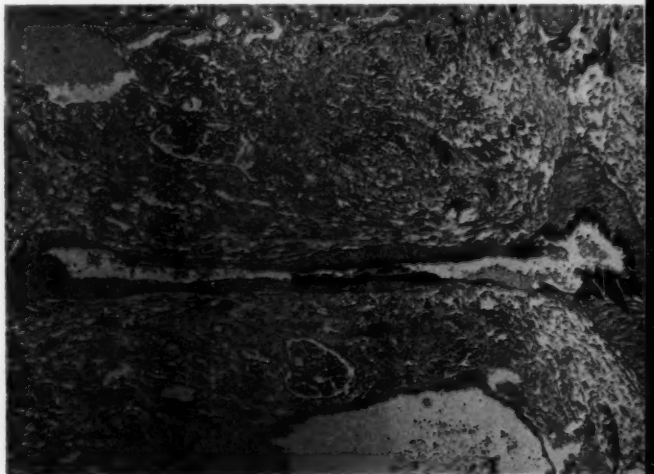
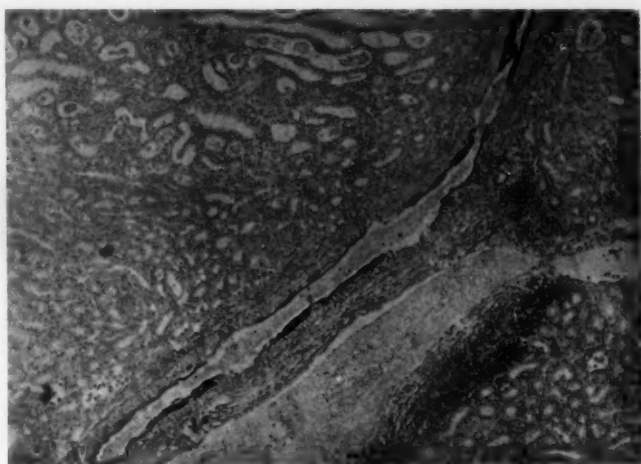


Fig. 27. — Rupture of internal elastic lamina in two segments of the artery; lumen not dilated. Two days after ligating ureter. Weigert stain;  $\times 65$ .

Fig. 28.—Rupture of internal elastic lamina in three places along an artery, with some fusiform dilatation at these sites. Six days after ligating ureter. Weigert stain;  $\times 65$ .



had destruction of both the media and the internal elastic lamina, the lumen was filled with a very fresh loose fibrin thrombus (Fig. 25).

*Time of Occurrence of the Ruptures.*—

The lesions are first seen at two days after the ureter is ligated. Once formed, they do not seem to undergo any further change during the next couple of weeks, and thus there is no satisfactory histologic method of assessing their age during that period. For example, in a kidney at 2 days, the ruptures look very fresh; but precisely similar appearances are seen in a kidney at 10 days and could well be interpreted as having occurred just before death. No information of value can be obtained from a study of the media and intima at the site, because these structures do not develop any inflammatory or reparative changes during the first two weeks.

Some evidence can, however, be obtained from the incidence of ruptures in the different experimental groups. If the animal has an intact contralateral kidney, the ruptures are seen first at three days, are present in about half the cases during the first week, and are found in all cases after that time. On the other hand, if the contralateral kidney has been removed a month or more previously, the ruptures are seen first as early as two days and are present in every

case at two and three days (these particular experiments cannot continue longer than three days). Intermediate results were obtained in the few cases in which the contralateral kidney was removed at the time that the ureter of the experimental kidney was tied; the ruptures were first seen at two days, but they were present in only about half the cases at two and three days.

It may be inferred that in animals with only a single kidney most of the ruptures occur at two to three days, and that in animals with an intact contralateral kidney the ruptures probably occur at intervals between the third and the seventh days. It is not impossible that some further ruptures may occur even later, but ordinary histologic examinations do not give the impression that the total number of ruptures in kidneys at 14 or 21 days is significantly greater than in kidneys at 7 days.

*Later Stages.*—The first signs of repair are seen at about two weeks, when a diffuse thickening of the connective tissue of the intima occurs in the region of the ruptures of the elastic lamina. During the next couple of weeks fine elastic fibrils are slowly laid down in this thickened intima, and these gradually become coarser during the next month. At this stage they sometimes condense to a single layer of elastica which crosses the gap in the original lamina, and

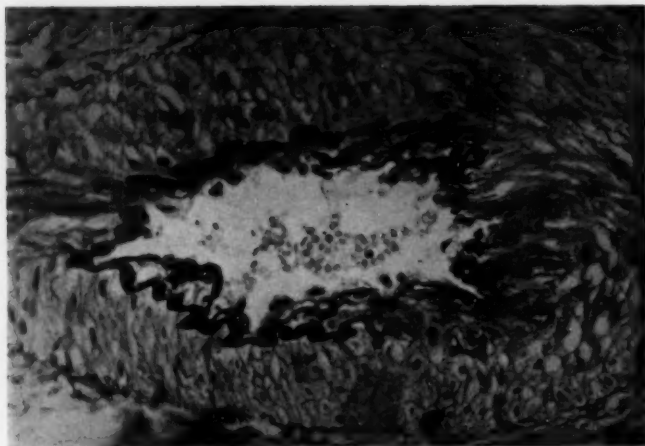


Fig. 29.—Healing stage. On the right there is formation of new elastic fibrils in the thickened intima at the site of rupture of the internal elastic lamina. Twenty-five days after ligating ureter. Weigert stain;  $\times 340$ .

extends rather a long way in the intima beyond, though it does not reestablish continuity with the broken ends of the old lamina (Fig. 29). In other cases the fibrils give rise to several thin layers of elastica, which have a loosely woven appearance (Fig. 30); this is well seen in parts of the artery where the original internal elastic lamina has totally disappeared (Fig. 31). The process of repair is very similar to that seen in the elastic tissue of the dermis in striae gravidarum.

#### Comment

Rupture of the internal elastic lamina is such a striking lesion that we have been

surprised to find only one description of it in the literature. Altschul and Fedor<sup>1</sup> described these ruptures in the human kidney in which hydronephrosis was present, and also in experimental animals in which the ureter had been ligated for periods of one week and upward. They do not appear to have investigated the condition earlier than a week. Fabian<sup>7</sup> has an illustration of what is obviously the healed stage of a rupture of the internal elastic lamina in prolonged experimental hydronephrosis, but he does not mention it in the text.

*In Vivo Nature of the Ruptures.*—The absence of aneurysms or of hemorrhage, the great rarity of thrombosis, and the lack of

Fig. 30.—Healing stage, showing formation of fine elastic fibrils around large gap in internal elastic lamina. Twenty-five days after ligating ureter. Weigert stain;  $\times 340$ .

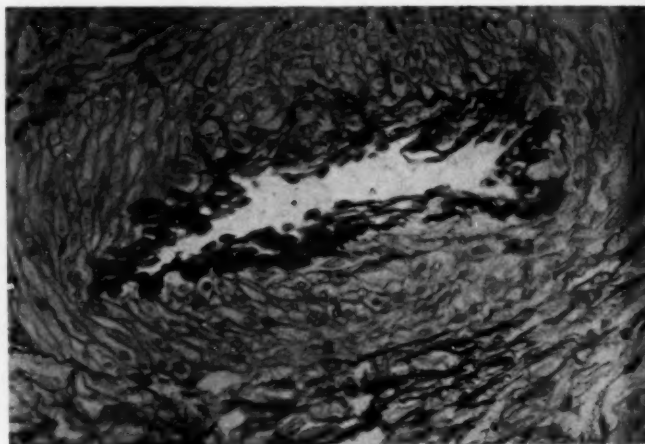
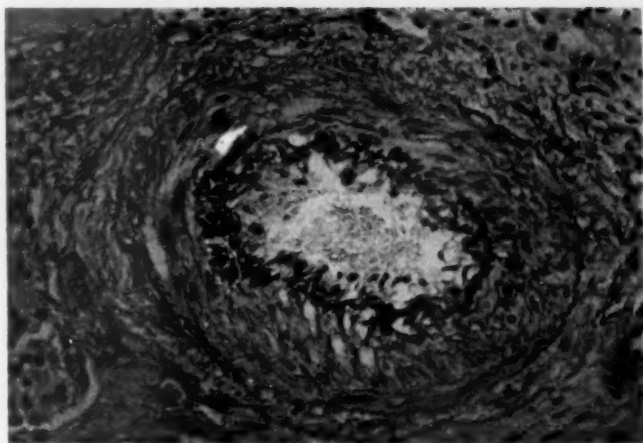


Fig. 31.—Repair stage of complete loss of internal elastic lamina. New elastic fibrils have been formed around the entire circumference. Twenty-five days after ligating ureter. Weigert stain;  $\times 340$ .



any reactive changes in the media or intima during the first week or so suggest at first sight that the rupture of the internal elastic lamina could be a peculiar form of post-mortem artifact, produced during the slicing of the kidney before fixation, and caused by the drag of the razor on the tissues.

An initial objection to this theory is that no ruptures of the internal elastic laminae have been found in a very large number of control kidneys without hydronephrosis, all of which had been sliced for fixation in the same manner as in the present series. It might, nevertheless, be argued that, in the special case of the hydronephrotic kidney, an abnormal fragility could develop in the elastic laminae of these arteries, so that they were more easily ruptured in the process of slicing before fixation. As against this, the ruptures are much less common at three days if the other kidney is intact than if it has been removed previously, though in both cases the experimental kidney has an obvious hydronephrosis.

Nevertheless, to test the possibility that the appearances might be due to a necropsy artifact, three further experiments were performed on animals which had had the left kidney removed a month previously. The ureter of the right kidney was ligated for three days. The kidney was then removed from the body with the greatest care to avoid any traction whatsoever on the

pedicle, and was fixed entire in formal-saline for three days before being sliced for histologic processing. These kidneys all showed numerous ruptures of the internal elastic lamina involving arteries in exactly the same sites as in kidneys sliced before fixation. Thus it cannot be accepted that the ruptures are artifacts produced by trauma at the necropsy.

The direct evidence that the lesions do occur *in vivo* is that repair begins about two or three weeks after the ureter has been ligated. The successive stages of this repair can be followed in the time series of the longer experiments, and the histologic appearances of repair are undoubtedly genuine.

*Mechanics of the Ruptures.*—There is some evidence that the most important factor which determines the occurrence of the ruptures is the absolute size of the kidney. The lesions are seen almost invariably if the kidney weighs 20 gm. or more after the pelvis is emptied. (a) In an animal with two kidneys, the experimental kidney reaches this weight at about three to seven days; the ruptures begin at three days and increase in frequency up to seven days. After that time the kidney always weighs 20 gm. or more, and ruptures of its elastic laminae are invariably present. (b) If the contralateral kidney has been removed a month previously, the experimental kidney

probably weighs about 10 gm. before the operation on the ureter. This hypertrophy involves an increase in the distance from the hilus to the fornices, and presumably this leads to a "taking-up of the slack" in the pelvic septa and their vascular bundles. When the ureter is ligated, the kidney enlarges rapidly and passes the critical weight by about two days. The already taut septa are further stretched, and thus ruptures of the elastic lamina of the arteries are found in every case of this group examined at two days. Furthermore, the ruptures are always numerous in such kidneys.

Some consideration must also be given to the view that the *rate* of dilatation of the pelvis might be just as important as the *extent* of this dilatation. The point at issue is that, if an animal has only one kidney, this kidney should be secreting urine at twice the normal rate; ligation of the ureter would thus be expected to produce a very rapid dilatation of the pelvis. This would account quite well for the early development of ruptures of the elastic laminae in animals that have had a previous unilateral nephrectomy. However, the theory does not provide a satisfactory explanation for the late development of the ruptures in all the animals with two kidneys; the pelvis is probably not dilating more rapidly at seven days than at two days.

The only reasonable explanation of the multiple ruptures of the internal elastic laminae in acute hydronephrosis is that they are produced by a longitudinal stretching of the arteries, though it is admittedly surprising that the intima and media are not also disrupted at these sites. The localization of the damage to the septal arteries is relatively easily understood. These arteries are attached at one end to the hilus and at the other end to the parenchyma at the fornix; this distance is rapidly extended when the pelvis enlarges. The tension along these septal arteries could obviously be transmitted for some distance along the arcuate arteries, producing the ruptures in these vessels.

The mechanical disturbances during the first few days seem to vary according to the different stresses and strains which develop in the vascular bundles of the septa as the pelvic cavity dilates. These will be of two types: first, a continuous steady traction, and second, abrupt alterations as the "guy ropes" (i. e., the arteries) stretch suddenly when the internal elastic laminae snap. The interference with the vessels should therefore be conceived not as a static process but, rather, as an irregularly progressive one.

#### Other Changes in the Arteries

The media of the arteries is usually normal apart from the lesions already noted as occurring in relation to the ruptures of the internal elastic lamina.

However, in about one-sixth of the cases there is a hyaline change in the media involving one, or sometimes two, septal or arcuate arteries in a single section. This consists of the deposition of a very refractile and strongly eosinophilic material in the media, sometimes restricted to the box walls between the media cells (as in the hyalin change produced by ischemia<sup>35</sup>), sometimes affecting the cells themselves but not the box walls, or sometimes forming a confluent mass replacing the whole structure. Occasionally the adventitia in the neighborhood contains a similar material or is infiltrated with red corpuscles. This change in the media may occur at any time after tying the ureter. It appears to be unrelated to the ruptures of the internal elastic lamina, and the two conditions often occur quite separately. Occasionally they both affect the same part of the artery, but there is no evidence that this is more than coincidental.

In the later stages of the hydronephrosis the cortical parts of the arterial tree become progressively stretched out and undergo a gradual atrophy. This has been demonstrated by Hinman and his co-workers<sup>20,21</sup> in gross injection specimens; it is not so easily recognized in ordinary histologic slides.

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Mention may be made here of two focal abnormalities which are found fairly commonly in the pelvic arteries of apparently normal rabbits; these must not be mistaken for pathologic lesions produced by experimental procedures.

(a) The large arteries in the septa not infrequently show peculiar irregularities of the media: In some areas the media may appear very thin or almost deficient; in other areas there is a wedge of media cells at right angles to the course of the other media cells. The same picture may be seen in the arteries of the hydronephrotic kidney, but is no commoner than in control animals.

(b) A brief reference has already been made to the structures called "elastic cushions." These cushions consist of a gross lenticular thickening of the deeper intima, produced by a pad of elastic fibrils parallel to the internal elastic lamina (Fig. 32). There is often a deficiency of muscle in the media nearby. They have been described by Müller<sup>31</sup> in man, chimpanzee, and pig, and even in human embryos. They are so common in normal rabbits that their presence in experimental material must be interpreted with great caution. Altschul and Fedor<sup>1</sup> observed these cushions in their long-term hydronephrotic kidneys and considered that they were due to the healing of tears of the elastic lamina. We do not accept

this view, because in our material the healing stages of ruptures of the lamina have a characteristic appearance which is quite different from that of elastic cushions. In the present experiments elastic cushions are no commoner at several weeks than at three days after ligating the ureter, and their incidence in the experimental kidneys is the same as in normal control kidneys.

### Veins

*Normal Histology.*—In the kidney of the normal rabbit the veins in the cortex and intermediate zone have a remarkably thin wall, consisting of little more than a layer of endothelium; they form rather irregular spaces, indented by the neighboring tubules, and their appearance is very similar to that of lymphatics in other parts of the body. The veins in the pelvic septa are much the same, though beneath the endothelium they have a thin layer of collagen with a few delicate elastic fibrils in it. It is only when these vessels are entering the hilar tunnel that they acquire a recognizable media.

*Hydronephrosis.*—There are no significant histologic lesions in the veins if the contralateral kidney is intact; this applies to all cases from one day up to four months after the ureter is tied. On the other hand, if the contralateral kidney has been removed, either at the time the ureter is ligated or

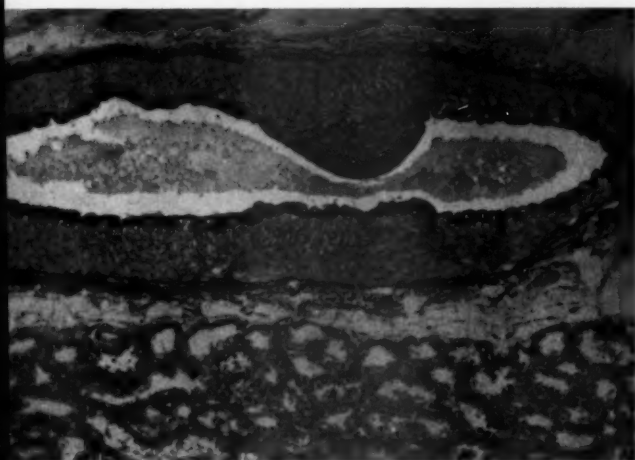


Fig. 32.—Elastic cushion in intima of an artery of the septum in a normal rabbit. Weigert stain;  $\times 120$ .

some weeks previously, the experimental kidney commonly shows thrombosis of a number of the septal veins at the fornix where they pass from the renal parenchyma into the septum (Fig. 1, *P*). The thrombosis is usually occlusive at one point, but does not propagate for more than a short distance along the length of the vein. The lesion is first seen at about 24 hours and is present in nearly half the cases at 2 and 3 days. By three days organization is developing, giving rise to clefts lined by endothelium. No later stages have been studied, because these animals with only one kidney have to be killed three days after the ureter is tied.

In the superficial cortex above these thrombi, but of course at a considerable distance from them, there are usually small patches of infarction or of ischemic damage.

A negative observation of some interest is that there are never any lesions in the walls of veins comparable to the tears of the elastic lamina in arteries. Presumably, the veins yield more easily to tension.

One further point may be mentioned because it has given rise to some confusion in the literature. In histologic sections of the hydronephrotic kidney, the veins in the parenchyma are usually filled with colloid protein instead of blood, or may contain a number of large mononuclear cells. Some red corpuscles usually remain in the larger veins in the septa. This histologic picture is certainly an artifact, due to the redistribution of fluids which occurs at the time of death.<sup>35</sup> It is an index of the amount of interstitial edema that was present in the kidney at the time it was removed, and in the acutely hydronephrotic kidney there is much edema of this type. The histologic appearances must not be taken as a direct indication of the renal circulation during life.

#### Comment

The thrombosis of the septal veins at the fornix is clearly an *in vivo* phenomenon. The vein at this site appears wide; this is because it is completely filled during life

by the thrombus, and thus cannot collapse after death in the way that a normal vein does.

There are two possible explanations for the thrombosis. First, there may be a relative slowing of the blood flow in this part of the vein if the parts nearer the hilus are narrowed. Second, the thrombosis may be related to the tears in the pelvic wall, assuming that these tears do, in fact, occur during life. The tears usually do not open into the veins themselves, but they cut across capillaries which drain into the veins after a very short course; any urine passing in through the tears might thus initiate thrombosis in the veins.

This second view finds some support in the fact that the thrombosis occurs only in animals that have had the contralateral kidney removed, so that the experimental kidney is attempting to handle the full load of excretion of urine. Furthermore, the thrombosis develops quite quickly after the ureter is tied; it begins at about 24 hours and is becoming organized as early as 3 days. This evidence suggests that the important factor giving rise to the lesion is the speed with which the pelvis becomes dilated. A rapid dilatation could produce acute stresses in the region of the fornix, and thus cause mechanical damage or tears at this site. It will be observed that this explanation is not the same as that given to account for the rupture of the elastic lamina of arteries. Those ruptures appear to be related essentially to the degree of dilatation of the pelvis rather than to the rate of the dilatation.

#### General Comment

At first sight the development of hydronephrosis might appear simple and self-evident: The obstruction causes an increase of pressure in the pelvis and throughout the nephrons, and this leads to a dilatation of the pelvis and to a pressure or disuse atrophy of the cortex. However, since the paper by Griffiths<sup>13</sup> in 1889, and even before that time, most of the workers who have studied the subject have been dissatisfied with this theory, and their experimental

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findings have led them to an explanation in terms of vascular disturbances. Our own findings point to the same conclusion. The general problems have been well reviewed by Hinman.<sup>18,19</sup>

Many of the individual points have been discussed in the comments as they arose; it remains now to correlate the more important of them.

There can be little doubt that, during at any rate the first few weeks after the ureter is ligated, the kidney continues to secrete urine into the pelvis. This is shown by the progressive dilatation of the pelvis and the upper segment of the ureter. The qualitative and quantitative aspects of this secretion have been discussed by a number of recent authors and will not be analyzed here.

Some of the urine escapes from the pelvis by pyelovenous or pyelointerstitial backflow. The route of the latter is marked by the edema of the pelvic septa and the hilar tunnel, and by the edema which spreads from the hilus around the kidney.

The amount of fluid which escapes in this way cannot be directly assessed, but, from experiments to be reported elsewhere, it can certainly be surprisingly large. Nevertheless, the pressure in the pelvis must clearly be maintained at a high level, because the pelvis undergoes a progressive dilatation. This dilatation gives rise to various effects.

1. It stretches the arteries of the pelvis, giving rise to multiple ruptures of the internal elastic lamina. The tension on the arteries also causes small areas of cytolytic necrosis under the arcuate fibrous bands.

2. There is evidence of impairment of the circulation through the kidney. Various grades of ischemic damage, up to full infarction, develop in patches in the cortex. This ischemia appears to be venous in origin because the lesions have the same characters as the ischemic lesions produced in the cortex when the renal vein is partially obstructed but the ureter is left open. The relationship of this venous obstruction to the dilatation of the pelvis is shown by the fact that the blood cannot escape freely from

the kidney while the pelvis is distended, either post mortem or in vivo.

3. The fluid pressure in the pelvis produces a cytolytic necrosis of the medullary papilla and of the summits of the peripelvic columns. The general obstruction of the renal circulation also plays a part.

These three points require some detailed consideration here. Their common factor appears to be the rise in pressure and the dilatation of the pelvis, and this acts indirectly by impairment of the renal circulation rather than by a direct obstruction of the outflow from the nephrons.

All the effects are increased if the contralateral kidney has been removed several weeks previously. Under these conditions the experimental kidney is hypertrophied at the time its ureter is ligated, and the vessels in the pelvis are already slightly stretched. The whole burden of attempted secretion falls on this kidney, and thus the pelvis dilates more rapidly. These two factors combine to accelerate the renal damage.

*Arteries.*—The rupture of the internal elastic lamina of the arteries is a very striking histologic feature and immediately raises the problem of the functional efficacy of these vessels. Their vasomotor control must presumably be affected by the extreme tension to which they are subjected. There is certainly a blood flow along them, because the kidney remains alive and continues to secrete urine. The remarkable absence of aneurysm formation at these weak points in the arterial wall may be because the raised pressure in the pelvis, and thus in the septa, may act as an external support to the weakened arterial wall, or the blood pressure may conceivably be reduced by some vasoconstriction of the main renal artery.

Several workers have studied the functional state of the arteries in the kidney after tying the ureter. Ricker<sup>33</sup> observed alterations in the responses to ether and epinephrine. Bianchi and dell'Adami<sup>4</sup> found by means of angiography that a narrowing of the renal arterial tree begins about a quarter of an hour after the ureter is tied, and con-

tinues at any rate for three weeks. These authors attribute the changes to vasoconstrictor reflexes from the dilated ureter. Deming<sup>6</sup> considered that the damage was ischemic and that the ischemia was due to a "shunt" mechanism; he based this explanation on the belief that the cortex atrophied more rapidly than the medulla, but in fact, as has been shown previously, the medulla disappears first.

Nevertheless, the ruptures of the elastica can occur without giving rise to any of the focal cortical lesions, such as infarcts. Furthermore, all the parenchymal lesions can be satisfactorily explained as due to venous obstruction. Thus we are driven to the tentative conclusion that these peculiar arterial lesions are of little functional significance. This, of course, does not exclude the possibility that there may be some reduction of arterial blood flow, particularly in the later stages.

*Veins.*—Although the veins show nothing comparable to the dramatic ruptures of internal elastic laminae, it seems probable that disturbances in them play a dominating part in the production of the parenchymal lesions in the kidney. The most direct evidence is the occurrence of thrombosis, but the indirect evidence is scarcely less convincing. The discussion here will be concerned with the mechanism of the venous obstruction.

The site of the impairment of the venous outflow has been the subject of much debate. Several workers have postulated that the renal veins are obstructed where they are stretched over the dilated extrarenal part of the pelvis. This may well be of significance in hydronephrosis in human beings. In the rabbit, however, the dilatation of the pelvis is entirely intrarenal, and there is no recognizable stretching of veins on the surface of the ureter where it enters the hilus.

The hilar tunnel is a very probable site of obstruction of the renal veins in the rabbit. The renal vessels and ureter lie in loose fat in this tunnel, whose walls consist of relatively firm cortex. The conditions are somewhat analogous to those at the neck of

a hernial sac. When the ureter is only slightly distended, the fat around it could buffer the pressure on the vein; but when the ureter is greatly enlarged, it will occupy most of the hilar tunnel and thus tend to compress the vein. This view is in accord with the observation that, after the kidney is removed, releasing the pressure in the ureter allows an immediate flow of blood from the vein.

The third possible site of obstruction is in the large veins in the septa. These veins, like the arteries, are attached at one end to the parenchyma at the fornix and at the other end to the hilar tunnel, and thus are subject to the same stresses when the pelvis dilates. In the absence of direct evidence, it is possible only to theorize about the mechanical conditions in these veins. The main stem of the veins in the septa might be narrowed as a result of the longitudinal tension, or it might be kinked at the places where tributary veins enter it along the subsidiary septa. In addition, the dilatation of the secondary pouches could compress the veins in the subsidiary septa between them or in the elongated terminal segments of the main vascular bundles. The peripheral end of the vein at the fornix is probably held open because of its attachments to the surrounding tissues; these are stretched apart by the expansion of the pelvis. This may play some part in determining the site of the thrombosis.

The fourth possible site of obstruction is in the veins of the renal parenchyma itself. Ferrer<sup>8</sup> says that the intralobular veins descending from the cortex join with the venae rectae ascending from the medulla and form a suprapylar plexus, from which the large interlobar veins arise. He postulates that in hydronephrosis the pyramid may be pushed up sufficiently to compress this suprapylar plexus. We have no evidence on this point.

Barney<sup>3</sup> and Lee-Brown<sup>23</sup> put the site of obstruction farther back, in the capillary bed. They suggest that the intertubular capillaries are compressed by dilatation of the tubules and by interstitial edema. This explanation

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is certainly not applicable to the cortex. During the first few days after the ureter is tied, the tubules are in fact never dilated in the areas where the evidence of ischemia is most obvious. Furthermore, there is evidence that the capillaries there are not compressed. At naked-eye observation during life, the surface of the kidney is not pale; in fact, its dark color indicates that the intertubular capillaries are as full of blood as in a kidney whose renal vein has been partially occluded. This is in agreement with the findings of Swann et al.<sup>30</sup> and of Gottschalk.<sup>12</sup>

On the other hand, there are good grounds for believing that the necrosis of the pyramid and the peripelvic columns is related to compression of the capillaries there. The cause of the compression can, however, not be accepted as an interstitial edema or a dilatation of tubules; it is clearly a result of the raised pressure in the pelvis.

*Late Stages.*—By two months the greater part of the cortex is reduced to fibrous tissue, and it might therefore be expected that little or no further secretion of urine would occur. Nevertheless, the pelvis does continue to dilate up to, at any rate, four months. This may be related to the few nephrons that retain their structural integrity or to some secretion from the lining epithelium.

The final atrophy of the cortex is presumably related to the prolonged interference with the renal blood flow.

A week or more after tying the ureter, the flow of blood through the main renal vein appears to be permanently impaired. Ghoreyeb<sup>10</sup> found that at this stage the obstruction to perfusion through the kidney persisted even after the dilated pelvis was punctured, so that the obstruction could no longer be ascribed to a simple compression of the vein by the dilated ureter in the hilar tunnel. As has been noted earlier, the collateral veins are sometimes enlarged at this time. Lindemann<sup>25</sup> and Barney<sup>2</sup> regard these collaterals as important and say that, if they are destroyed, the kidney atrophies and the pelvis does not enlarge any further.

Scott<sup>34</sup> does not agree with this. The subject is rather obscure and requires further investigation.

## Summary

Progressive hydronephrosis was produced in rabbits by permanent ligation of the ureter. The experiments were performed on three groups of animals. In the first group the contralateral kidney was left intact; in the second it was removed at the time of the experiment, and in the third it had been removed several weeks previously.

The renal pelvis dilates continuously during the first few weeks. The weight of the kidney increases by about three times, because the cortex, though thinned, is expanded to a large area.

The proximal tubules show a transient dilatation during the first two or three days, and thereafter slowly atrophy. The broad ascending limbs and distal convoluted tubules undergo a progressive dilatation and finally becomes greatly enlarged. Some interstitial fibrosis develops toward the end of the first week. After a few months the cortex consists mainly of fibrous tissue with dilated lower segments in it, but it also contains a small number of proximal tubules with relatively normal appearance.

In addition, there are a series of overtly ischemic focal lesions, ranging from small wedges of congestion and necrosis of proximal tubules up to frank infarcts. These are most marked in the deep cortex and intermediate zone, but may spread down into the medullary pyramid.

There is a progressive ulceration of the medullary pyramid; the necrosis begins at the tip of the papilla and steadily progresses more deeply, so that after a few weeks the entire pyramid is eroded away. This necrosis is of "cytolytic" type and is due to ischemia produced by the raised pressure in the pelvis. A similar cytolytic necrosis occurs along the summits of the peripelvic columns, but this heals without ulceration. Small areas of similar necrosis occur under the arcuate fibrous bands because of the localization of pressure at this point.

Gross edema of the pelvic septa occurs, and the edema fluid exudes from the hilus out into the perirenal tissue.

The arteries in the pelvis commonly develop multiple ruptures of the internal elastic lamina within the first week. This appears to be due to a stretching of the arteries; they are anchored at one end to the hilar tunnel and at the other end to the renal parenchyma, so that, as the kidney enlarges the two ends are pulled apart. The repair processes do not begin until about three weeks; they consist of the laying down of elastic fibrils in the intima, but the original elastic lamina is not reconstituted.

In the group of animals in which the contralateral kidney has been removed several weeks previously, the lesions are severer than in the other two groups. The ruptures of the internal elastic laminae occur earlier; the veins are often thrombosed at the pelvic fornice; frank infarcts are confined to this group, and the less severe grades of focal ischemic lesions are rather common.

The findings throw some light on the mechanisms whereby the various parenchymal lesions are produced. Obstruction to the outflow of urine from the nephrons does not seem to play an important part. Obstruction to the venous drainage is perhaps the most significant factor. The necrosis of the pyramid is partly due to direct pressure by the urine in the pelvis which compresses the capillaries. The site of obstruction of the venous drainage is probably the hilar tunnel, where the renal vein is compressed by the dilated ureter. There may also be a vasomotor disturbance in certain of the damaged arteries.

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# Effect of Subcellular Fractions of Ascites Tumor Cells on Tumor-Host Relationship in Mice

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## Introduction

Fractions of various ascitic fluids have been shown to contain a factor lethal to mice.<sup>1</sup> If such a factor originates in the ascites tumor cells, it is possible to envisage its release *in vivo*, probably under conditions adverse to some portion of the ascites-cell population. Information about the effects of such a subcellular component on the tumor-host system is thus relevant to any complete picture of the changes in that system resulting from the introduction of carcinostatic agents. This paper presents the results of studies of the effects on normal and tumor-bearing mice of a variety of subcellular fractions made from ascites tumor cells.

## Materials and Methods

**The Tumor and Mice.**—An Ehrlich ascites EF hypodiploid tumor was used in these studies. The origin and history of this tumor are given elsewhere.<sup>2</sup> The tumor is carried in white male Swiss mice by weekly intraperitoneal transfer of approximately  $10 \times 10^6$  tumor cells. The growth and lethality of this tumor have been described in detail elsewhere.<sup>2,3</sup>

***In Vivo* Experimental Procedures.**—Fresh tumor was removed from stock transfer animals and the cells separated from the ascitic fluid by gentle centrifugation. The tumor cells were then resuspended in 0.9% NaCl and injected into fresh animals at the various dosages specified below. In experiments in which a tumor-cell fraction was to be tested, a solution of that fraction was substituted in place of the 0.9% NaCl as the inoculum

medium. In the experiments described in Tables 3 and 5 the tumor cells were not separated from their original ascites fluid. The sizes of an experimental group was 12 animals unless otherwise noted.

**The *In Vitro* Experimental Procedure.**—The trypan-blue staining of ascites cells has been correlated with viability.<sup>4</sup> Viable cells display an impermeability to this stain. The *in vitro* experimental procedure employed was as follows: An aliquot of fresh tumor cells suspended in 0.9% NaCl at a concentration of  $40 \times 10^6$  T (tumor) cells per milliliter was prepared and incubated at 37 C. At various times after the start of the incubation two 1 ml. samples of incubated cells were withdrawn and separately diluted 1:9 in 0.9% NaCl. Each sample was then treated in the following manner: One milliliter of the sample was added to a vial containing 1 ml. of 1% trypan blue and 2 ml. of 0.9% NaCl. The final concentration of tumor cells was thus about  $1 \times 10^6$  cells/ml. After 10 minutes the number of cells stained in the sample was counted in a hemocytometer. Thus, for each time interval in an incubation experiment, duplicate samples were obtained.

In experiments designed to test the effect of various tumor-cell fractions on the viability of whole tumor cells *in vitro*, the whole cells were initially suspended in a solution of the tumor-cell fraction being tested and then incubated. Samples of these incubation mixtures were withdrawn and tested with trypan blue, as described above.

**Cell Fractionation Procedures.**—Table 1 presents an outline of the three main fractionation procedures utilized in the preparation of the subcellular extracts. The fractions resulting from these procedures are designated, respectively, by the Roman numerals I, II, and III. As shown in Table 1, all three preparations involved final centrifugations; the subscripts "s" and "p" are used to denote, respectively, the fractions taken from the centrifugal supernatants and those taken from the precipitate. The concentrations of the various fractions are given in Table 1 as whole-cell equivalent concentrations. Thus, for example, Fraction I<sub>s</sub> contains, per milliliter, that amount of cellular material of its type that would be contained in  $20 \times 10^6$  whole cells per milliliter.

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TABLE 1.—Cell Fractionation Procedures

Fraction Type	Procedural Steps Followed	Equivalent Concentr.	Fraction Symbol
I* Mechanical fractionation in 0.9% NaCl	(1) 10 min. shaking with glass beads	$95 \times 10^4$	
	(2) Quick freeze—thaw 6 times, dilution in 0.9% NaCl	$20 \times 10^4$	
	(3) Centrifugation 1 hr., 14,000 g		
	Supernatant	$20 \times 10^4$	I <sub>s</sub>
	Precipitate (resuspended in 0.9% NaCl)	$20 \times 10^4$	I <sub>p</sub>
II Mechanical fractionation in 0.22% NaCl (0.25 isotonic)	(1) 10 min. shaking with glass beads in 0.9% NaCl	$72 \times 10^4$	
	(2) Addition of distilled H <sub>2</sub> O; quick freeze-thaw 5 times	$18 \times 10^4$	
	(3) Elimination of heavy cell fragments obtained as precipitate from centrifugation 20 min., 6,000 g		
	(4) Centrifugation 1 hr., 14,000 g		
	Supernatant	$26.8 \times 10^4$	II <sub>s</sub>
	Precipitate (resuspended in distilled H <sub>2</sub> O)	$26.8 \times 10^4$	II <sub>p</sub>
III Autolysis in 0.9% NaCl	(1) Incubation 24 hr. at 37 C in 0.9% saline	$40 \times 10^4$	
	(2) Centrifugation 1 hr., 14,000 g		
	Supernatant	$40 \times 10^4$	III <sub>s</sub>
	(3) Heat III <sub>s</sub> to 100 C 15 min.		
	(4) Centrifugation 10 min., 1,460 g		
	Supernatant	$40 \times 10^4$	III <sub>s-1</sub>
	(5) Fraction III <sub>s-1</sub> precipitated with 76% alcohol at 0 C, 50 min.		
	(6) Centrifugation 15 min., 1,460 g		
	Supernatant (lyophilized, resuspended in distilled H <sub>2</sub> O)	$40 \times 10^4$	III <sub>s-2</sub>
	(7) Fraction III <sub>s-2</sub> centrifuged at 14,000 g 15 min.		
	Supernatant	$40 \times 10^4$	III <sub>s-3</sub>

\* There was also a Fraction I<sub>sp</sub>, which was prepared simply by freezing and thawing a whole-cell suspension of  $31 \times 10^4$  cells per cubic centimeter twice. It is classed with Fraction Type I because the fractionation was performed in 0.9% saline.

## Results

*The in Vivo Experiments.*—Two types of survival criteria are employed in evaluating the in vivo experiments. Tables 2 to 6 show the percentage of animals in the experimental groups dead after two, four, five, or six days, as specified, while in Table 7 the mean survival time of the experimental group is recorded. Tables 2 and 3 give the results of experiments in which the cell fractions were injected intraperitoneally into tumor-free mice. Autopsy of surviving mice revealed no tumor in any of the experimental groups. It is apparent from Tables 2 and 3 that cell Fraction I<sub>p</sub>, the saline extraction precipitate; I<sub>sp</sub>, the mixed saline fraction, and III<sub>s</sub>, the autolysis supernatant, contained factors which killed experimental animals within a few days after inoculation, whereas other fractions tested did not.

The lethal activity of the centrifugal precipitate fraction, I<sub>p</sub> (Table 2), indicates

that the responsible substance is localized in the cell fragments; and the negative results with Fractions II<sub>s</sub> and II<sub>p</sub>, the supernatant and precipitate, respectively, from the extraction in 0.22% NaCl, are therefore explicable by the assumption that the lethal factor was discarded with the heavy cell fragments after the preliminary low-speed centrifugation in the preparation of these fractions (Table 1). However, after autolysis, the lethal factor is found in the supernatant phase.

TABLE 2.—Effect of Mechanically Extracted Tumor-Cell Fractions (Types I and II) on Mice

Fraction	No. of Animals in Experiment Group	Per Cent of Animals in Experiment Group Dead by Day		
		2	5	6
I <sub>s</sub>	12	0	0	0
I <sub>p</sub>	12	0	17	25
I <sub>sp</sub>	8	0	13	38
II <sub>s</sub>	12	0	0	0
II <sub>p</sub>	12	0	0	0

TABLE 3.—Effect of Tumor-Cell Autolysate Fractions (Type III) on Mice

Fraction	Group	No. of Animals in Expt. Group	Inoculum Strength, Expressed in Terms of Equivalent Numbers of Whole Cells/Inoculum	Per Cent of Animals in Experimental Group Dead by Day		
				2	5	6
III <sub>a</sub>	10		60×10 <sup>4</sup>	20	50	50
III <sub>a</sub>	6		20×10 <sup>4</sup>	33	83	--
III <sub>a</sub>	6		8×10 <sup>4</sup>	0	50	--
III <sub>a</sub>	6		4×10 <sup>4</sup>	17	50	--
III <sub>a</sub> -1	22		40×10 <sup>4</sup>	0	0	0

Table 4 presents the result of *in vivo* experiments in which the cell fractions were used as suspended media for an inoculum of 10×10<sup>6</sup> fresh tumor cells. The last column gives the comparable per cent of animals dead at six days, already recorded in Tables 2 and 3 and obtained from experiments in which only the respective cell fractions were injected into tumor-free mice.

Table 5 presents a further series of similar experiments with Fraction III<sub>a</sub> in which the concentration of the cells incubated in the preparation of this fraction and the number of whole cells inoculated with the cell fraction were varied.

In an additional experiment, in which Fraction III<sub>a</sub>-1 (heat-treated autolysate) was inoculated with 10×10<sup>6</sup> fresh tumor cells, 25% of the animals died in seven days, while the control group, into which an equal amount of Fraction III<sub>a</sub>-1 was injected without whole cells, showed no deaths after seven days. Groups treated with doses of 10×10<sup>6</sup> fresh cells in saline

TABLE 4.—Effects of Tumor-Cell Fractions Inoculated with 10×10<sup>6</sup> Whole Tumor Cells\*

Cell Fraction Inoculated	Cell-Fraction Concentration in Terms of Equivalent Number of Whole Cells/Inoculum	Per Cent of Animals Dead by Day			Per Cent of Tumor-Free Animals Dead by Day 6, from Tables 2 and 3
		2	5	6	
I <sub>a</sub>	20×10 <sup>4</sup>	8	8	17	0
I <sub>b</sub>	20×10 <sup>4</sup>	25	50	50	25
II <sub>a</sub>	26.8×10 <sup>4</sup>	17	33	42	0
II <sub>b</sub>	26.8×10 <sup>4</sup>	0	0	0	0
III <sub>a</sub> (21 animals)	40×10 <sup>4</sup>	45	45	62	50†
Saline control (40 animals)	--	0	2.5	5	--

\* Twelve animals per experiment.

† This figure represents per cent of animals dead from an inoculum of Fraction III<sub>a</sub> equivalent to 60×10<sup>4</sup> whole cells. No experiment using Fraction III<sub>a</sub> at the equivalent concentration of 40×10<sup>4</sup> cells/inoculum was recorded in Table 3.

routinely had no more than 5% dead after seven days.

By introducing the cellular autolysate fractions *in vivo* along with a very small dose of fresh tumor cells, any inactivation of the latter might show up as failure of tumor take in the experimental animals. The dose chosen was 0.4×10<sup>6</sup> cells/ml/animal, and the fractions employed were III<sub>a</sub>-1 and III<sub>a</sub>-2. Table 6 records several such experiments, and also two experiments in which the autolysate fraction was injected intraperitoneally 24 hours after the tumor inoculation. The control groups, totaling 32 animals, were inoculated with 0.4×10<sup>6</sup> cells/ml/animal in saline. Autopsies re-

TABLE 5.—Effect of Varying Inocula of Tumor Cells with Fraction III<sub>a</sub> of Varying Equivalent Concentration\*

Concentration of Cells Incubated in Preparation of the Autolysate	No. of Whole Tumor Cells Inoculated	Per Cent of Animals Dead After Four Days
40×10 <sup>4</sup>	40×10 <sup>4</sup>	75
40×10 <sup>4</sup>	10×10 <sup>4</sup>	49
40×10 <sup>4</sup>	1.5×10 <sup>4</sup>	33
10×10 <sup>4</sup>	40×10 <sup>4</sup>	33
10×10 <sup>4</sup>	10×10 <sup>4</sup>	17
10×10 <sup>4</sup>	1.5×10 <sup>4</sup>	0
1.5×10 <sup>4</sup>	10×10 <sup>4</sup>	0

\* Twelve animals per experiment.

TABLE 6.—Inhibition of Tumor Take by Heat-Treated and Cold-Alcohol-Precipitated Autolysate Supernatants (Fractions III<sub>a</sub>-1 and III<sub>a</sub>-2)

Time of Treatment	Fraction	No. of Animals in Group	Per Cent Tumor Take (Saline Control 65.4%)
Inoculated with tumor	III <sub>a</sub> -1	12	50
	III <sub>a</sub> -1	20	53
	III <sub>a</sub> -2	20	23
Inoculated 24 hr. after tumor inoculation	III <sub>a</sub> -1	12	33
	III <sub>a</sub> -2	12	11

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vealed a mean incidence of tumor growth in 65% of these animals.

Four days after the inoculation of  $1.5 \times 10^6$  tumor cells per animal, the average total population of cells in intraperitoneal suspension was  $72.6 \times 10^6$  cells per animal.<sup>3</sup> About half this number of cells was withdrawn from a fourth-day animal and injected into another fourth-day animal. If the cells were injected in minimal fluid volume so as to increase the intraperitoneal cell concentration, a dramatic increase in lethality results (Exper. 1, Table 7). That this effect is dependent on the increase of cell concentration, and not on number, is shown by the results of Experiment 2 in Table 7, where the same number of cells was added in a larger fluid volume. No increase in lethality was found. Now, if this same number and concentration of fourth-day cells are fractionated into Fraction  $I_{sp}$  before inoculation, the increased lethality found in Experiment 1 again occurs (Exper. 3). Quantitatively similar results are obtained if tumor-free animals, instead of fourth-day tumor-bearing animals, are treated with a similar inoculum of Fraction  $I_{sp}$  (Exper. 4). The interpretation of these effects is of interest and is brought out in "Comment."

TABLE 7.—Mean Survival Times of Groups of Tumor-Bearing Animals Inoculated on Fourth Day of Tumor Growth with Added Tumor Cells and Cell Fraction  $I_{sp}$

Experiment	Fourth-Day Inoculation	Volume of Fourth-Day Inoculation, Ml.	No. of Animals	Mean Survival Time, Days
1	$31 \times 10^4$ whole cells	Minimal	24	7.25
2	$31 \times 10^4$ whole cells	1.0	10	17.25
3	$I_{sp}$	1.0	10	9.0
4	$I_{sp}$ (injected into tumor-free animals)	1.0	12	8.5
Control	$31 \times 10^4$ cells/animal in saline	--	10	16.75
Control	$1.5 \times 10^6$ cells/animal in saline	--	32	17.25

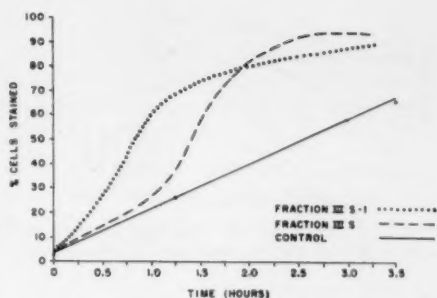


Fig. 1.—Per cent of cells stained with trypan blue plotted vs. time.

*The in Vitro Experiments.*—The in vitro experiments with the cell fractions, in which trypan blue was used as a viability indicator, are summarized in Figure 1. The control points represent the percentage of tumor cells staining blue after various periods of incubation in 0.9% saline at 37 C. In preliminary experiments based on the control curve, the general validity of the trypan-blue technique as a viability criterion was verified for this tumor. Thus, for example, after 1.5 hours of incubation about 27% of the cells were stained; after 3 hours, 60% of the cells stained, and after 7 hours 100% of the cells stained. Experiments in which cells incubated 1.5, 3.0, and 7.0 hours were washed and injected with  $10 \times 10^6$  cells per animal resulted, respectively, in tumor take in 100%, 75%, and 0% of the experimental animals. While the cells which stain are thus apparently inviable, it is concluded for the following reason that some nonstaining cells have also become nonviable: Using fresh tumor cells, a 100% take is commonly observed with doses smaller than the number of unstained three-hour cells injected, which is on the average  $4 \times 10^6$  cells/inoculum. Since only 75% take was observed with this many three-hour cells, staining with trypan blue can be considered to be a very conservative estimate of the cellular damage which results in loss of viability.

Figure 1 demonstrates that when both cell Fractions  $III_s$  and  $III_{s-1}$ , the heat-treated autolysis supernatant, replaced 0.9%

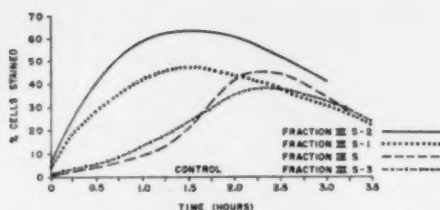


Fig. 2.—Per cent of cells stained blue with trypan blue plotted vs. time and vs. the control.

NaCl as the suspension medium for the ascites cells, the rate of appearance of inviable cells was greatly increased. Comparative results of similar experiments with tumor-cell-autolysate Fractions III<sub>a</sub>-2 (cold-alcohol treated) and III<sub>a</sub>-3 (centrifuged Fraction III<sub>a</sub>-2 material) are shown in Figure 2, plotted as differences from values shown in the control curve. Rate of appearance of inviable cells increases progressively from Fraction III<sub>a</sub> to Fraction III<sub>a</sub>-2, probably owing to the successive purifications of the responsible substances. The preparation of Fraction III<sub>a</sub>-3 from III<sub>a</sub>-2 by high-speed centrifugation, however, decreased the effectiveness of the latter fraction by approximately one-half. It would thus seem that the tumor-cell-inactivating factor is localized, in part, in the particulate matter centrifuged out of Fraction III<sub>a</sub>-2. This material is apparently characterized by heat stability and relative solubility in both water and cold alcohol.

### Comment

It is clear that the tumor cells contain a substance lethal to mice, and that this substance is released when the cells are crudely fractionated. Presumably the destruction of living tumor cells in the animal has a lethal effect, similar to that of the tumor-cell fractions prepared from artificially destroyed cells. This interpretation was, in fact, suggested by the early occurrence of deaths among tumorous animals treated with carcinostatic ovarian extracts in this laboratory.<sup>2</sup> The trypan-blue experiments presented in Figures 1 and 2 demonstrate the presence of an anti-tumor-cell activity

in the tumor-cell-autolysate fractions themselves. When an autolysate fraction is injected into mice along with a sufficient number of living tumor cells, it may be expected that the anti-tumor-cell factor would damage the tumor cells, with consequent release of additional lethal factor. On this basis, the death rate of the animals should increase (relatively) if the concentration of the cells autolyzed to produce the pertinent cell fraction is increased or if the number of living cells injected along with the autolysate is increased. Table 5 presents data supporting these assumptions. Table 4 can now be interpreted on similar grounds: Fraction II<sub>a</sub>, for example (which in the absence of whole tumor cells was not harmful to the experimental animals), is thought, similarly, to have attacked the living tumor cells inoculated with it, and thereby to have caused *in vivo* the release by these cells of the lethal substances which were responsible for the death of 42% of the animals by the sixth day. Thus there is an indirect, as well as a direct, lethal effect on the mouse.

That the factors responsible for these two types of action are physically separable is shown in Table 4 for Fractions I<sub>a</sub>, II<sub>a</sub>, and II<sub>b</sub>, and by the difference in results obtained with the inoculation of Fraction III<sub>a</sub>-1 (heat-treated autolysate) in the presence and in the absence of living tumor cells. All four of these fractions possess the capability of causing the release of a factor from whole tumor cells, presumably by destroying or damaging them. However, in the absence of whole tumor cells they are completely harmless to the mice into which they are injected (Column 6, Table 4). The anti-tumor-cell action of certain of the extracts is further demonstrated by their inhibition of tumor development in animals given low initial doses of tumor cells.

In a separate paper, we have discussed the relationship between the fluid volume of this ascites tumor and the number of tumor cells actively maintained in suspension in that fluid.<sup>3</sup> It was pointed out there

that during the early days of tumor growth (one to six days) there are present in the peritoneal cavity a number of tumor cells not suspended in the ascites fluid. These cells may be brought into suspension merely by the addition of fluid volume. If the unsuspended state is selectively deleterious to the cells, the early deaths of some host animals might be attributed to the elaboration of a lethal factor from them. It will be remembered that when cells are injected into mice which have carried a tumor for four days the concentration of cells in the inoculum is important. The same number of cells in higher concentration is more lethal than in lower concentration. If the above reasoning is correct, the lethality of the higher concentration is to be correlated with the assumption that the increase of intraperitoneal cell concentration would induce more cells to settle out. If, instead of adding live cells, equivalent concentrations of fragmented cells are introduced in either the presence or the absence of living tumor cells, a similar lethality results. Our general conclusion is that the death of the animals in these experiments is due to a factor elaborated from deteriorating unsuspended cells; the elaboration of this factor is functionally analogous to the artificial destruction of tumor cells in the preparation of the cell fractions effective in Tables 2 and 3.

### Summary

Subcellular fractions made from ascites tumor cells by fragmentation and by autolysis contain a substance lethal to mice. This substance is heat-labile.

These tumor-cell fractions also contain a factor which attacks whole tumor cells in vitro. This factor is not heat-labile.

An interpretation of the role played by these factors in the deaths of host animals under conditions adverse to the tumor-cell population is discussed.

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# Further Studies of the Carcinostatic Effect of Extracts Prepared from Cow Ovaries

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It has been suggested that the rate of cell division, indeed cell division itself, is to some extent dependent on the balance of heparin-like anticoagulant substances and their antagonists. The abnormal rate of division observed in malignant cells has been attributed to the lack of, or dysfunction of, one of the components of this regulatory balance.<sup>3</sup>

The general concept stated above was the basis of the work of which this paper constitutes the second part. In the mammalian ovary there is a marked regulation of cell division, and in cow ovaries it has been shown that there is a high concentration of anticoagulant substances.<sup>2</sup> In a paper published recently, various types of extracts made from cow ovaries were tested on mice inoculated with a lethal ascites tumor.<sup>8</sup> The antimetabolic action of such extracts has been reported previously.<sup>4,5</sup> The results of this work indicate that from cow ovaries there may be extracted a substance(s) that has a carcinostatic effect on the lethality of an ascites tumor in mice.

In the work reported here, another method of extraction is tested in terms of the effect of the extract on tumor-bearing mice. Also, the extracts, "active" and "inactive," are tested as to their effect on the multiplication of ascites tumor cells *in vivo*.

## Materials and Methods

*Serial Aqueous Extraction.*—Whole cow ovaries were obtained and cleaned.\* The connective tissue

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\* Ovaries were obtained from the slaughterhouse of the Cross Brothers Meat Packing Co. The ovaries were processed immediately after being obtained.

was trimmed away from each ovary and the resulting material ground in a meat grinder, and then weighed. Ground ovarian material was extracted in a solution of 0.9% NaCl which contained 100 units of penicillin and 50  $\mu$ g. of streptomycin per milliliter of extraction medium. The extraction ratio used in these preparations was 2 ml. of extraction medium to 1 gm. of ovarian material. This mixture was allowed to extract for one hour in a cold room at 4 C. After this period the mixture was centrifuged for 30 minutes at a speed of 2,000 rpm in a refrigerated centrifuge. The supernatant solution was decanted and stored at 4 C. The remaining solid material was then reextracted for one hour under the same conditions as those described above. This mixture was, in turn, centrifuged and the resulting supernatant extract stored at 4 C. The solid material remaining after this second extraction was then reextracted for a third time, as described above. Thus, three supernatant extracts were obtained from one lot of ovarian material. They were designated as I, II, and III.

The three supernatant extracts were then individually precipitated by the addition of cold ethanol to a final concentration of 70%. The precipitates obtained were removed from the respective mixtures by centrifugation, lyophilized, and stored as powders in a desiccator. The extraction procedure outlined above was performed on several lots of ovarian material.

*Purification of Crude Extracts.*—The crude preparations obtained by extraction (to be designated as the "crude" fraction) were further purified. The powdered material was placed in a solution of 3 parts ethanol to 1 part ether (50 ml. of solution per gram of extract) and kept at a temperature of 45 C for 30 minutes. The mixture was centrifuged and the supernatant discarded. This procedure was repeated three times. Subsequently, the precipitated material was further treated by placing it in a solution of 1 M NaCl for one hour at 100 C (50 ml. of solution per gram of material). The mixture was then centrifuged and the supernatant solution discarded. This procedure was repeated with 30 ml. of solution per gram of material. After the second wash in 1 M NaCl solution, the mixture was centrifuged and the precipitate washed with water. The solid material remaining was then lyophilized. In the subsequent discussion this fraction is designated

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as the "protein" fraction. The above procedures were designed to rid the extract of loosely bound lipids and nucleic acids.<sup>6</sup> Extracts I, II, and III were each purified in the manner described. For each extract there was, then, a "crude" and a "protein" fraction.

*The Mice and the Tumor.*—The Ehrlich ascites EF hypodiploid tumor was used in these studies. The origin and history of this tumor are given elsewhere.<sup>6</sup> The tumor is carried in white male Swiss mice by weekly intraperitoneal transfer of approximately  $10 \times 10^6$  tumor cells. The dosage used in the survival experiments was  $1.5 \times 10^6$  tumor cells. In these survival experiments, 24 hours after the intraperitoneal inoculation of  $1.5 \times 10^6$  tumor cells in 0.2 ml. of 0.9% NaCl, treatments with the extract to be tested were begun. Each tumor-bearing animal received a daily intraperitoneal inoculation of 15 mg. of dry extract suspended in 0.5 ml. of 0.9% NaCl for a period of five days. In the case of the purified fractions of the extracts, the "protein" fractions were suspended in 0.5 ml. of distilled water. The treated animal therefore received a total of 75 mg. of extract in a period of five days.

Mice inoculated with tumor and treated with extract were observed for 30 days. After this time, the surviving mice were killed, and an autopsy was performed to determine the presence of tumor cells or of a solid growth in the peritoneal cavity. In this manner, both the crude and the protein fraction of hourly serial extracts of the ovarian material were tested for any effect they might have on the survival time of the tumor-bearing mice.

*Growth of the Tumor in Vivo.*—We have studied the growth of this ascites tumor when injected into white Swiss mice at a dosage of  $1.5 \times 10^6$  tumor cells in 0.2 ml. of 0.9% NaCl. Groups of animals were inoculated with the tumor cells and subsequently killed on given days after inoculation. The total number of ascites cells in suspension and the fluid volume of the tumor were determined by a dye-dilution method adapted from Révész and Klein.<sup>7</sup> The animal to be killed was injected with a known amount and concentration of the dye sulfobromophthalein sodium (Bromsulphalein sodium; BSP). The dye was allowed to mix in the animal for six minutes, after which the animal was killed by severing the spinal cord in the region of the neck. Two aliquots of fluid containing cells were removed from the peritoneal cavity of the dead animal by syringe. In one of these aliquots the concentration of ascites tumor cells was determined by counting the cells in a hemocytometer. The second aliquot was placed in a hematocrit tube and centrifuged at a force of 1,347 g for 15 minutes. This procedure was used to determine the ratio of cells to fluid in the peritoneal exudate. The supernatant fluid in the hematocrit tube was

removed after centrifugation. A portion of this fluid was made 0.06 N with respect to NaOH and the optical density of the mixture determined in a Bausch & Lomb Spectrophotometer at a wave length of  $580\mu$ . The ascites-fluid volume of the tumor was then determined by means of the following relationship:

$$\frac{OD_{std}}{OD_{tinal}} \times \frac{\text{Vol. of dye injected}}{V_1} = V_1$$

in which  $V_1$  is the total ascites-fluid volume in the tumor plus dye,  $OD_{std}$  is the optical density of a known concentration of the dye and  $OD_{tinal}$  is the optical density of the dye diluted by the fluid which was originally present in the peritoneal cavity of the tumor-bearing animal. The original ascites-fluid volume of the tumor is then obtained by subtracting the volume of the dye sulfobromophthalein injected from the total volume figure ( $V_1$ ) obtained from the above relationship. The total number of cells suspended in the peritoneal exudate is then determined by the relationship

$$\text{Total number of cells} = C \left[ V_1 \left( \frac{H}{1-H} \right) + V_1 \right]$$

in which  $C$  is the concentration of ascites-tumor cells per millimeter as determined in the hemocytometer,  $V_1$  is the volume of the ascites fluid plus the dye injected,  $H$  is the proportion of the peritoneal exudate plus dye that is cells, and  $1-H$  the proportion that is fluid.

The total number of cells in suspension and the ascites-fluid volume of the tumor in the peritoneal cavity of the mice was determined on the 2d, 4th, and 5th through the 10th day of tumor growth. Owing to the slight amount of fluid in the tumor, on the second and fourth days, 3 ml. of dye solution was injected into each test animal; on the fifth and sixth days, 2 ml. of dye was injected, and on the final days, only 1 ml. of the dye solution was used.

*Procedure for Testing Effect of Extracts on Multiplication of Ascites Cells.*—A group of animals was inoculated with tumor at a dose of  $1.5 \times 10^6$  cells in 0.2 ml. of 0.9% NaCl. On the third day of tumor growth a number of animals were selected at random; half of these animals received an intraperitoneal injection of the extract to be tested; the other half were injected (I. P.) with 1 ml. of 0.9% NaCl. Some of the animals in the control group were killed one-half hour after the injection of the saline in order to determine what effect the increase in volume of the tumor would have on the number of cells suspended in the peritoneal exudate. On the fourth day of tumor growth, i. e., 24 hours later, all animals in both groups were killed, and the total number of cells and the fluid volume of the tumor were determined. Likewise, on the fifth day of tumor growth a group of animals were injected with 60 mg. of dry extract suspended in 2 ml. of saline and

killed on the sixth day (24 hours later). This procedure was repeated with another group of animals on the seventh day of tumor growth. In this case the experimental animals received a dose of 120 mg. of dry extract suspended in 3 ml. of saline.† In both the fifth- and the seventh-day experiments, the control animals were injected with similar volumes of 0.9% NaCl. Some of these animals were killed one-half hour after injection in order to determine the effect of the volume change on the number of cells suspended in the peritoneal fluid. This procedure was designed to obtain some index of the effect of the extracts on the multiplication of the tumor cells during the 24 hours following the injection of the test extract.

Extracts were classed as "active" and "inactive" on the basis of their effect on the survival of the tumor-bearing mice. It has been reported previously that the extract obtained by extracting ovarian material in a basic medium (pH 12) had no effect on the survival time. Purified fractions of this extract were likewise ineffective.<sup>8</sup> In the experiments described above this extract was used as the "inactive" extract, and the "protein" fractions of the serial extracts were used as the "active" extracts.

† All the animals were killed on the eighth day and the total cells and fluid volume per animal were determined as in the previous two cases.

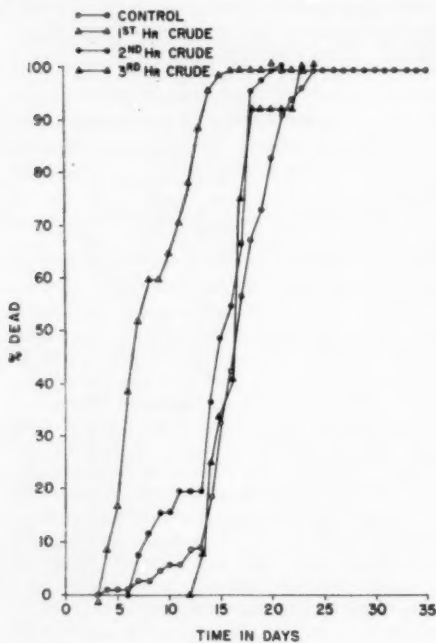


Figure 1

## Results

**Survival Experiments (Series 1).**—In six separate experiments in which mice were inoculated with the test dose of tumor, an average of 99.9% of the control animals had died by the end of the 30th day. A total of 124 animals were used in the six control groups. The cumulative mean per cent of dead mice with respect to time, in days, is shown in Graphs 1 and 2. These values were derived on the basis of the fact that the per cent of animals dead on the same day in six separate control groups is binomially distributed. The mean survival time of a control group is 17.1 days, with a standard error of 0.7 days ( $17.1 \pm 0.7$ ).

The results of the study of the effect of serial extracts of ovarian material on the survival of the tumor-bearing mice are illustrated in Table 1 and Graphs 1 and 2. The crude preparations of the first, second, and third hours of extraction had no effect on the survival of tumor-bearing mice. The purified fractions (the "protein" fractions) of all three serial extracts, however, had an effect on the survival of the tumor-bearing mice. In animals treated with the "protein"

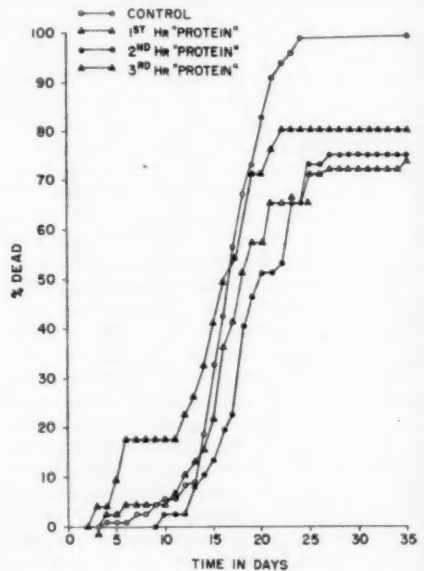


Figure 2

## EXTRACTS PREPARED FROM COW OVARIES

TABLE 1.—Survival of Tumor-Bearing Mice Treated with Ovarian Extracts

Extract	Fraction	Average per Cent of Mice Surviving After Day				Total No. of Animals
		10	15	20	30	
I (1st hr.)	Crude	35.2	1.8	0.1	0.01	24
	Protein	95.6	78.2	42.5	25.7	46
II (2d hr.)	Crude	84.4	51.7	0.1	0	25
	Protein	97.2	86.2	48.7	24.7	36
III (3d hr.)	Crude	100	67	8	0	12
	Protein	82.3	58.7	28.5	19.7	24
Control		94.3	67.7	17.0	0.1	124

fractions of the first and second hours of extraction there were averages of 25.7% and 24.7% surviving at the termination of the experiments. These values are significantly different from those of the control series at the 0.01 level of probability. The significance of differences in per cent dead on the 30th day was derived from the confidence-coefficient tables of Clopper and Pearson.<sup>1</sup> Animals treated with the "protein" fractions of the third-hour extract showed a survival of 19.7% at the termination of the experiments. This value is not significantly different from that of the control at the 0.01 level of probability, but is at the 0.05 level. In the case of surviving animals treated with the first-hour "protein" fractions, 63.6% had no apparent tumor and 36.4% were found to have solid growths in the peritoneal cavity when autopsy was performed. Surviving animals treated with second-hour "protein" fractions showed on autopsy 70% with no apparent tumor and 30% with solid growths in the peritoneal cavity. The surviving animals treated with the third-hour "protein" fractions showed on autopsy 75% with no apparent tumor and 25% with solid growths in the peritoneal cavity. The mean survival times of the three groups of animals treated with "protein" fractions of the first-, second-, and third-hour extracts were  $16.9 \pm 1.8$ ,  $18.9 \pm 1.4$ , and  $14.1 \pm 1.7$  days, respectively. None of these values is significantly different than the mean survival time of the control animals at the 0.01 level of probability.

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TABLE 2.—Growth of the Untreated Ascites Tumor

Day of Tumor Growth	Avg. Total Cells $\times 10^4$ per Animal	Standard Deviation $\times 10^4$	Avg. Fluid Vol. (ml.) per Animal	$g_t$ of Cells, Hr.	No. of Animals
2	7.8	4.8	0.23	20.3	8
4	72.6	52.3	0.61	15.0	11
5	205.6	141.3	1.2	16.0	9
6	314.9	160.8	1.7	39.3	10
7	633.3	136.3	2.4	24.1	8
8	827.1	342.1	4.0	62.8	11
9	963.6	125.8	4.2	109.5	10
10	1,074.4	284.2	6.8	155.4	9

*Growth of the Tumor.*—The results of the study of the growth of the tumor in the peritoneal cavity of the mouse following the inoculation of  $1.5 \times 10^6$  tumor cells in 0.2 ml. of 0.9% NaCl are summarized in Table 2 and in Graphs 3-6. Eleven separate inocula of the test dose of tumor and a total of 77 animals were used to determine the various parameters of the growing tumor. Table 2 summarizes, for the days of tumor growth listed, the average total number of tumor cells suspended in the ascitic fluid per animal, the average fluid volume of the peritoneal exudate per animal, and the mean generation time ( $g_t$ ) of the average population of tumor cells during the interval of time ending with the day on which the figure is listed. The  $g_t$  is calculated on the basis of the relationship

$$g_t = \frac{t}{3.3 \log \frac{b}{B}}$$

where  $B$  is the total number of tumor cells in suspension at the beginning of the interval of time;  $b$ , the number of tumor cells at the end of the interval of time, and  $t$ , the interval of time, in hours.<sup>6</sup> The value of  $g_t$  is, then, the time, in hours, that it would take the average population of tumor cells to double if the rate of multiplication remains the same as that of the average population of cells during the interval of time observed. The standard deviation about the mean total number of tumor cells per animal is listed in Table 2 as an indication of the variation of the total number of tumor cells from animal to animal on any one day of tumor growth.

Graph 3 shows the relation between the average number of tumor cells per animal, the average ascites-fluid volume of the tumor, and the time, in days, of tumor growth. After the 10th day of tumor growth, the average number of tumor cells per animal levels off at the value given for the 10th day and then decreases, whereas the fluid volume of the tumor continues to rise.<sup>8</sup> This phenomenon is probably an artifact, for the total number of tumor cells in the animal probably continues to increase. Observation of the peritoneal cavity of tumor-bearing animals after the 10th day of tumor growth reveals that the tumor cells

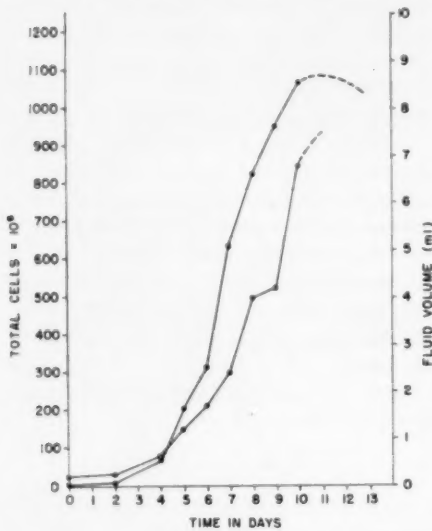


Figure 3

tend to adhere to the walls of the cavity and clump in large masses in the ascitic fluid, both of which factors would reduce the measured number of tumor cells in suspension in the ascitic fluid. Shortly after the 10th day of tumor growth there is a sharp increase in the rate of death of the tumor-bearing animals. Determinations of the total number of tumor cells in suspension and the fluid volume of the tumor are, therefore, somewhat impractical.

Graphs 4-6 illustrate some additional aspects of the growth of the tumor. The

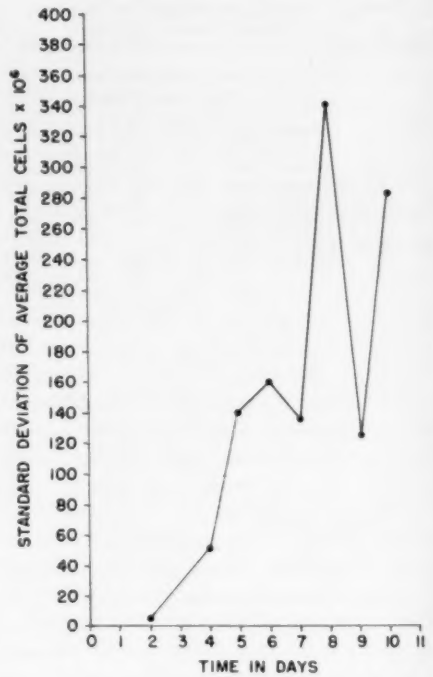


Figure 4

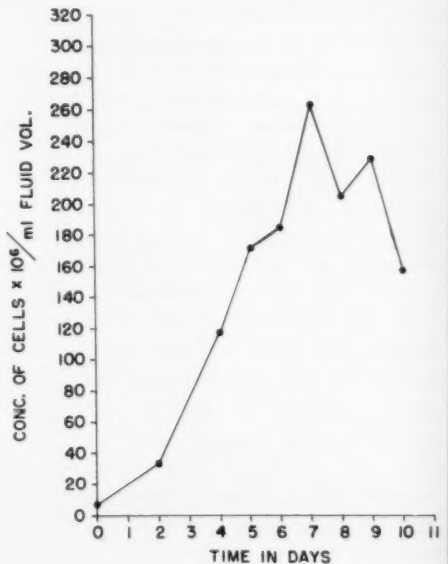
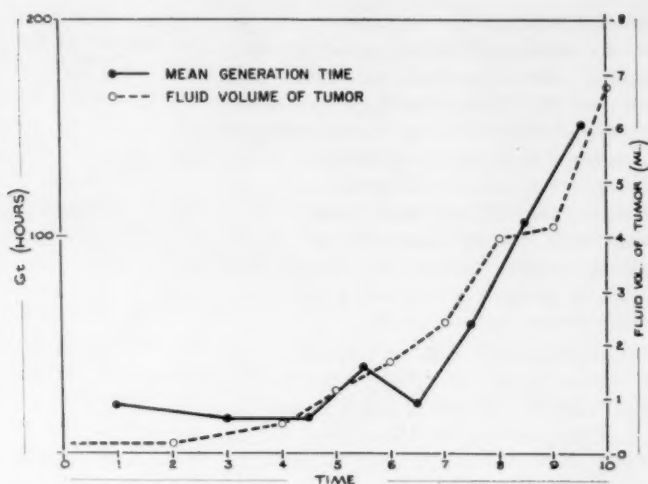


Figure 5

Fig. 6.—Mean generation time of cells *vs.* time; fluid volume of tumor *vs.* time.



variability of the total number of tumor cells suspended in the ascitic fluid from animal to animal on any one day increases with time, as is shown in Graph 4. This variability has a peak around the eighth day of tumor growth, after which it decreases. The average concentration of the tumor cells in suspension in the ascitic fluid per millimeter per animal increases until about the seventh day of tumor growth and subsequently declines (Graph 5). The mean generation time increases relatively slightly for the first 5 days of tumor growth; this is followed by a sharp increase in  $g_t$  through the 7th to the 10th day (Graph 6). The sharp increase in the  $g_t$  of the average population of tumor cells from the 7th to

the 10th day of tumor growth can again be attributed in part to the removal of cells from suspension in the ascitic fluid by adhesion and clumping.

*Effect of Extracts on Multiplication of Tumor Cells.*—The results of experiments designed to test the effect of the extract on the multiplication of ascites tumor cells *in vivo* are summarized in Tables 3, 4, 5, and 6. Table 3 shows that the tumor-bearing control animals inoculated with similar amounts of saline on the third and fifth days of tumor growth and killed one-half hour later gave greater, though not significantly greater, average total numbers of tumor cells in suspension per animal. On the seventh day tumor-bearing control animals had a smaller

TABLE 3.—Effect of Ovarian Extracts on Growth of the Tumor-Cell Population During the Intervals of the Third to Fourth, Fifth to Sixth and Seventh to Eighth Days Following Tumor-Cell Inoculation

Day	Control Cells $\times 10^4$	Exper. Control Cells $\times 10^4$ *	Sig. Diff.	Active Extract $\times 10^4$ *	Sig. Diff.	Inactive Extract $\times 10^4$ *	Sig. Diff.	Active <i>vs.</i> Inactive Sig. Diff.
3	23.8 †	34.8 (9)	0					
4	72.6	133.5 (14)	+	100.1 (9)	0	191.1 (6)	0	--
5	205.6	266.1 (9)	0					
6	314.9	503.0 (15)	+	322.7 (9)	--	639.3 (5)	0	--
7	633.3	443.5 (5)	0					
8	827.1	777.0 (9)	0	508.1 (6)	0	818.4 (2)	0	0

\* Numbers in parentheses show number of animals. Plus (+) or minus (–) signs indicate values to be significantly different at 0.01 level of probability; 0 indicates values are not significantly different at 0.01 level of probability.

† Calculated value.

average total number of tumor cells per animal, but not significantly smaller. Tumor-bearing control animals inoculated with saline on the third and fifth days of tumor growth and killed on the fourth and sixth days, respectively, had a significantly greater average total number of tumor cells per animal at the 0.01 level of probability. The same result was not observed in the tumor-bearing control animals inoculated with saline on the seventh day and killed on the eighth day.

Animals treated with "active" fractions of extract on the third, fifth, and seventh days showed a reduced average number of tumor cells per animal when killed on the fourth, sixth, and seventh days, respectively, as compared with the control animals on those days. However, only in the case of the cells tested on the interval of the fifth to the sixth day was that reduction significant at the 0.01 level of probability. Likewise, animals treated with "inactive" extract fractions on the third, fifth, and seventh days all showed an average total number of tumor cells per animal on the fourth, sixth, and eighth days, respectively, which was greater than that of the control animals on those days. None of these differences is significant. The "active"-extract-treated animals, as compared with the "inactive"-extract-treated animals, showed a significant reduction in the average total number of tumor cells per animal on both the fourth and the sixth day. In the experimental period of the seventh to the eighth day, while there is a sizable reduction in the average number of tumor cells per animal, this difference is not significant. The increased variability in the number of tumor cells per animal on the eighth day in this case makes it difficult to demonstrate a statistically significant difference, even though the difference is rather large.

Tables 4, 5, and 6 give more detailed outlines of the data obtained for the test of the effect of the extract on the ascites cells between the third to fourth, fifth to sixth, and seventh to eighth days of tumor growth.

TABLE 4.—Effect of Ovarian Extracts on the Growth of the Tumor-Cell Population During the Interval of the Third to the Fourth Day of Tumor Growth

Group	Avg. Total Cells $\times 10^4$ per Animal		
	3d Day	4th Day	#, Hr.
Control	23.8 *	72.6	15.0
Experimental control	34.8	133.5	12.5
Active extract		100.1	15.9
Inactive extract		191.1	9.8
Avg. Fluid Volume of Tumor per Animal, ml.			
	3d Day	4th Day	
Control	0.42 *	0.61	
Experimental control	0.92	0.81	
Active extract		0.84	
Inactive extract		1.2	

\* Calculated value.

The figures in the top half of the Tables deal with the changes in the average total number of tumor cells per animal during the interval of days of tumor growth cited, in the normal growth curve, the experimental control animals, and the animals treated with "active" as well as "inactive" extracts. In Table 5, for example, the inoculation of 2 ml. of 0.9% NaCl into the tumor-bearing control animals on the fifth day results in an immediate rise in the average total number of tumor cells per

TABLE 5.—Effect of Ovarian Extracts on the Growth of the Tumor-Cell Population During the Fourth to the Fifth Day of Tumor Growth

Group	Avg. Total Cells $\times 10^4$ per Animal		
	5th Day	6th Day	#, Hr.
Control	205.6	314.9	39.3
Experimental control	266.1	503.0	26.3
Active extract		322.7	87.1
Inactive extract		639.3	19.1
Cells added to control	268.1 *	524.6	24.9
Avg. Fluid Volume of Tumor per Animal, ml.			
	5th Day	6th Day	
Control	1.2	1.7	
Experimental control	2.5	3.0	
Active extract		3.2	
Inactive extract		4.5	
Cells added to control	1.7 *	2.2	

\* Calculated value.

animal of 29% over the average number of tumor cells normally found on that day of tumor growth. This 29% increase in cells in the ascitic fluid is followed by an even larger increase in number of cells in the average population on the sixth day, and this increase is definitely significant. Considered as a population, the tumor cells in the experimental control animals have a  $g_t$  of 26.3 hours, whereas the population of cells in the control animals has a mean generation time of 39.2 hours, on the basis of this same interval of 24 hours of tumor growth. In order to clarify further the situation in the experimental control animals, the following experiment was performed: Tumor was allowed to grow in a group of animals until the fifth day. On the fifth day some of these animals were killed and the tumor removed. These fifth-day tumor cells were then counted and injected into the remainder of the fifth-day animals in an amount calculated to increase the cellular population by roughly 30%, on the average. This inoculation was done in a minimal volume of fluid (0.5 ml.). These animals were killed 24 hours later, and determinations were made of the average number of tumor cells and the average fluid volume of the tumor per animal. The results of this experiment are listed at the bottom of the

first part of Table 5. The case of the added cells mimicked that of the experimental control animals, in which only saline had been added. In the control animals the immediate increase over the normal growth curve of 29% of the cell population is increased to an excess of 66.6% on the sixth day. The  $g_t$  values for these two groups were, therefore, very similar (Table 5).

The results in the case of the tumor-bearing animals treated with "active," as well as "inactive," extracts, as listed in Table 5, are self-explanatory. The  $g_t$  value for the average population treated with "inactive" extract is 19.1 hours. The bottom half of Table 5 deals with the volume changes in each of the cases considered in the top half of the Table. Animals treated with "active" extract showed an increased ascites-fluid volume on the sixth day of about 7% over that of the experimental control animals on the same day. However, animals treated with "inactive" extract showed an increase of 50% over that of the control animals on the sixth day.

### Comment

The effective fractions of the aqueous extracts were obtained by removing the loosely bound lipids and nucleic acids from the crude extracts. This material was extractable from the ovarian tissue in as short a time as one hour. Subsequent hourly extraction of the same material likewise gave active fractions. The "activity" of each of the three "protein" fractions was characterized by the survival of a percentage of treated tumor-bearing animals at the end of 30 days, a survival rate that was significantly different from that of the control group at the 0.05 level of probability. In the case of the "protein" fractions of the first- and second-hour extracts, the difference was significant at the 0.01 level of probability.

It was reported previously that the fraction of the aqueous extract precipitated by the addition of cold ethanol to a final concentration of 45% was "inactive," whereas that fraction precipitated by the further

TABLE 6.—Effect of Ovarian Extract on the Growth of the Tumor-Cell Population During the Interval of the Seventh to the Eighth Day of Tumor Growth

Group	Avg. Total Cells $\times 10^4$ per Animal		
	7th Day	8th Day	$g_t$ , Hr.
Control	833.3	827.1	62.8
Experimental control	443.6	777.0	29.9
Active extract		508.1	123.7
Inactive extract		818.4 *	27.3
	Avg. Fluid Volume of Tumor per Animal, Ml.		
	7th Day	8th Day	
Control	2.4	4.0	
Experimental control	4.5	5.3	
Active extract		5.7	
Inactive extract		3.1 *	

\* Small sample, only two animals.

addition of alcohol, from a concentration of 45% to a final concentration of 70%, did have an effect on the survival of tumor-bearing mice. In the work reported here, the crude fractions of the aqueous extract were precipitated by the addition of cold alcohol to a final concentration of 70%. These preparations had no effect on survival until the loosely bound lipids and nucleic acids were removed by purification methods described in "Materials and Methods." This point substantiates the idea expressed previously that the materials removed do in some way mask the effect of the extract on the tumor and suggests that these materials are largely to be found in the portion of the aqueous extract precipitated by the addition of cold ethanol to a concentration of roughly 45%.

It has been observed in this laboratory that after the 10th day of tumor growth the cells in the peritoneal exudate tend to clump in masses, as well as to deposit in large amounts on the walls of the peritoneal cavity. These two processes tend to reduce the measurable number of ascites tumor cells suspended in the ascitic fluid. The fall in concentration of tumor cells after the eighth day of tumor growth may be attributed to the continued increase in the fluid volume of the tumor and to the loss of cells from the population, due to the beginning of the process cited above.

The calculation of the mean generation time is dependent on the total number of tumor cells suspended in the ascitic fluid at the beginning and at the end of the time interval, on the basis of which the  $g_t$  is calculated. The fact that the average number of tumor cells per animal rises with time and levels off at a peak value is the apparent cause of the sharp increase in the  $g_t$  from the 7th through the 10th day of tumor growth. In a sense, the rise in the  $g_t$  is an artifact, for it does not give the mean generation time of the cells actually in suspension. All that one can say about the calculated  $g_t$  is that it is a function of the relation between the forces removing cells from the measured population in suspension

and the true  $g_t$  of the cells remaining in suspension in the peritoneal exudate. The possibility remains that the  $g_t$  of the cells remaining suspended in the ascitic fluid is fairly constant throughout the first 10 days of tumor growth.

The restriction of the nutritional uptake of the tumor cells by the animal would be expected to slow down the rate of multiplication of the suspended tumor cells, but whether such a restriction is imposed to any great extent during the first 10 days of tumor growth is a question. If during this period of time an ever-increasing proportion of the nutritional intake of the animal goes to the tumor, the animal will die if either of two alternatives ensues: first, if the amount of nutritional intake going to the tumor precludes utilization of the animal's requirements; or, second, if the amount of nutritional intake supplied to the tumor is not sufficient to keep the tumor cells alive. For the products of dead, cytolized tumor cells are toxic to the tumor-bearing animal. This latter conclusion is based on experiments done in this laboratory.<sup>10</sup> It was observed that mechanically broken-up tumor cells, as well as the products of autolyzed tumor cells, are toxic to tumor-bearing animals during a period of tumor growth when only a few animals ordinarily die as a result of the presence of the tumor. We believe that the calculated value of  $g_t$  rises during the first 10 days of tumor growth, as a result largely of the processes removing cells from suspension in the ascitic fluid, and that the rise is not due primarily to the restriction of the nutritional uptake of the tumor cells in suspension by the needs of the animal as a whole.

On the basis of the data presented in this paper and of the above assumption, it appears that from the time of inoculation of the tumor cells some cells are merely deposited out of suspension. Up until the fifth day of tumor growth, these cells, when brought back into the suspension by increasing the volume of the tumor, multiply even more rapidly than the cells already suspended in the peritoneal exudate. This is

illustrated by the experimental control average population of tumor cells, as noted in the study of the effect of the extract on tumor growth on the third to fourth days and on the fifth to sixth days of tumor growth. On the seventh day, the cells are no longer brought into the suspension by the injection of saline into the tumor-bearing animal, despite the fact that the loss of cells from the population due to adhesion to the walls of the peritoneal cavity and subsequent infiltration is increasing. The cells leaving the population appear now to be adhering more firmly to the cavity wall than during the previous days of tumor growth.

The calculated  $g_t$  of the tumor cells in suspension in the peritoneal exudate apparently depends during the first five or six days of tumor growth, when the cells not actively in suspension do not tend to adhere to the walls of the cavity so firmly, on the number of cells actively in suspension in the ascitic fluid, which is, in turn, dependent on the amount of volume supplied to the tumor by the animal. After the seventh day of tumor growth the calculated  $g_t$  depends more and more on the increasing number of tumor cells leaving the suspended population to adhere more firmly to the walls of the peritoneal cavity. The true  $g_t$  of the cells remaining in the suspension on the 7th through the 10th day of tumor growth may be as low, or nearly as low, as that calculated for the cells in the earlier intervals of time.

One factor or a combination of several factors may account for the reduction in the average number of tumor cells per animal in suspension in the ascitic fluid when the cells are treated with "active" material, as compared with "inactive" material, or as compared with the experimental control population of tumor cells. The cells could be destroyed, inhibited from dividing, or removed from the measurable population of tumor cells in suspension in the peritoneal exudate by the acceleration of such a phenomenon as adhesion to the cavity walls. In the absence of thorough histologic studies, the latter possibility cannot be ruled out.

The data on the volume changes of the tumor in the treated and experimental control populations of tumor cells suggest that the "active" material is utilized in some manner and the "inactive" is not. Hence the average population of tumor cells treated on the third and fifth days of tumor growth with the "inactive" material showed excess volumes of ascites fluid on the fourth and sixth days of 50% and 48%, respectively, over those of the experimental control population. In the same interval of time the population of tumor cells treated with the "active" material showed percentage ascites-fluid volume increases over those of the experimental control tumor of 4% and 7%, respectively. The percentage volume increases over the experimental controls on the seventh and eighth days was not too significant, for the sample was rather small.

### Summary

The carcinostatic effect of an aqueous extract of cow ovaries has been studied. The effect of this extract was measured in terms of the survival of mice inoculated with an Ehrlich ascites tumor and subsequently treated with the extract.

Control animals inoculated with  $1.5 \times 10^6$  tumor cells in six separate groups totaled 124. The mean survival time of such a control group was  $17.1 \pm 0.7$  days.

Tumor-bearing animals treated with crude fractions of three serially made aqueous extracts showed no significant survival at the end of 30 days.

Tumor-bearing animals treated with the so-called "protein" fractions of three serially made aqueous extracts showed a significant survival of 25.7%, 24.7%, and 19.7%, respectively, at the end of 30 days.

The mean survival time of a group of treated animals did in no case differ significantly from the mean survival time of the control animals.

The growth of the ascites tumor used in this work has been studied. The various parameters of the growth of the tumor are reported here.

The effect of extracts on the multiplication of the ascites cells during 24 hours between the third and fourth, fifth and sixth, and seventh and eighth days of tumor growth was studied.

Active extract injected on the third, fifth, and seventh days of tumor growth reduced the average total number of tumor cells per animal on the fourth, sixth, and eighth days, respectively.

Inactive extract injected on the third, fifth, and seventh days of tumor growth increased the average total number of tumor cells per animal on the fourth, sixth, and eighth days, respectively.

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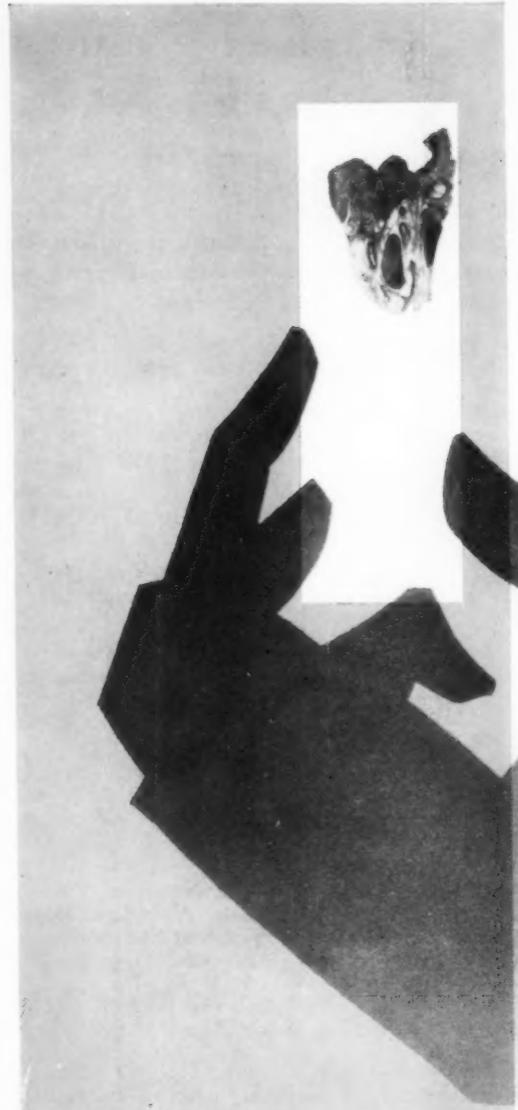
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